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Studies on Isoamylase.

Part VIII. On the Determination of Isoamylase Activity.[*]

By Tsuneo Kobayashi

Department of Agricultural Chemistry, University of Tokyo

Received May 12, 1955

A method for determining the isoamylase activity is devised. A properly diluted enzyme solution is allowed to act at 20°C on a 1% solution of glutinous-rice starch buffered to pH 6.2. After 24 hours, 0.2 cc of the reaction mixture is mixed with 2 cc of 0.01 N I_2 solution, and diluted to 25 cc. Optical density (E) of the solution at 620 mμ is read in a Pulfrich photometer, using a 1-cm cell. The increment of E at 620 mμ is proportional to enzyme activity within certain ranges. The amount of enzyme is expressed in the "isoamylase unit", i.e., an increase of E 0.200 being taken as 10 units.

A pH-activity curve is given for yeast isoamylase, which shows a sharp maximum at pH 6.2.

In the previous investigations on isoamylase, the author[1)] has established the action of this enzyme. The enzyme attacks starch-type polysaccharides of branched structure, such as glutinous-rice starch, amylopectin, or glycogen, at their branching points, forming a product of amylose-like character. This mechanism has been further confirmed by the end-group assay on both the substrate and product[2)], and has already received general approval[3)].

In the course of further investigations on purification and properties of the enzyme, it was necessary to establish a method to determine the enzyme activity with sufficient accuracy. The isoamylase activity has hitherto been estimated only qualitatively by visually observing the iodine coloration of the reaction mixture, which changes from red or brown into a bluish color when the enzyme acts on glutinous-rice starch or glycogen. Few attempts have been made on the quantitative treatment of this action. The author has, therefore, intended to devise a reliable method for its determination, suitable for routine work.

Two specific changes occur on the substrate properties during the isoamylase action, which may be utilized for the determination. The first, is the increase in the β-amylolysis limit, and the second, the variation of iodine coloration following enzyme action. When amylopectin or glycogen is attacked by isoamylase, the conversion limit by β-amylase is markedly elevated, as a result of the scission of branching linkages caused by the isoamylase. It would, therefore, be reasonable to design a method for determination on the basis of this fact, provided that a substrate of uniform molecular weight and uniform degree of branching be always available. But obviously, such a substrate will not always be accessible. Moreover, the method implies some further limitations. Whether the linear component of starch is completely hydrolyzed by β-amylase is a matter of discussion[4], and even if this question be overlooked, high accuracy will not be expected with the method, especially in the presence of those substances which affect reducing sugar determination. Several complications of the procedure, such as that it requires at least two determinations of reducing sugar in a single assay, or the needs of a powerful pure β-amylase preparation, make the method more difficult to be carried out.

---

[*] Preceding communications on the same subject: Part I, *J. Agr. Chem. Soc. Japan*, **23**, 115 (1949); Part II, *Ibid.*, **23**, 120 (1949); Part III, *Ibid.*, **24**, 309 (1951); Part IV, *Ibid.*, **24**, 347 (1951); Part V, *Ibid.*, **25**, 34 (1951); Part VI. *Nature*, **167**, 606 (1951); Part VII, *J. Agr. Chem. Soc. Japan*, **27**, 599 (1953).

1) T. Kobayashi et al., *J. Agr. Chem. Soc. Japan*, **23**, 115, 120 (1949); **24**, 309, 347 (1951); **25**, 34 (1951); *Nature*, **167**, 606 (1951).

2) T. Kobayashi and M. Kadowaki, *J. Agr. Chem. Soc. Japan*, **27**, 599 (1953).

3) e.g., E. J. Hehre, in F. F. Nord (ed.), "Advances in Enzymology", Vol. XI, Academic Press Inc., 1951, p. 326; W.Z. Hassid, in W.D. McElroy and B. Glass (Eds.), "Phosphorus Metabolism", Vol. I, Johns Hopkins Press, 1951, p. 21.

After examining the method based on the change of β-amylolysis limit, the author has set it aside for a while, and turned over to the second possibility, which is based on the variation of iodine reaction. It is a specific and remarkable action of isoamylase to change the iodine coloration of the substrate from red or brown into a bluish color. If the change is quantitatively measured under specified conditions, values corresponding to the enzyme activity may be obtained. In order to obtain a linear relationship between the coloration and enzyme activity, the author has studied various conditions using glutinous-rice starch as a substrate, and has devised a method of determination as described below. As the method is based on iodine coloration, specific to the starch-type polysaccharides, it is hardly affected by other substances. The application of colorimetry has raised the accuracy of results, and rendered simplicity of procedures. The method is satisfactorily reliable in routine determinations.

Experimental and Results

1. Procedures.

The maximum of the light absorption curve of glutinous-rice starch-iodine complex lies in the vicinity of 460 mμ. When isoamylase acts on the glutinous-rice starch, a product with the absorption maximum at 620 mμ appears, and increase in the absorption at 620mμ is approximately proportional to the enzyme action. In the presence of a large amount of unchanged substrate, the maximum at 620 mμ is clearly discernible only with low iodine concentrations. But the coloration is relatively unstable at low iodine concentrations, and an appreciable error may occur when the enzyme preparation contains such substances which react with iodine. In practice, therefore, the colorimetry is performed with a large excess of iodine. Though maximum absorption under this condition remains at 460~530 mμ, the increment of absorption at 620 mμ is proportional to the enzyme amount. The procedure described below is adopted according to the considerations mentioned above and also for convenience in operation.

Five cc of a 1% aqueous solution of soluble glutinous-rice starch (Lintner method) was buffered to pH 6.2 by adding 1 cc of a 0.5 N acetate buffer solution (The buffer may be previously included in the substrate solution). A properly diluted enzyme solution and water were added to make the total volume 7 cc. The mixture was allowed to react at 20°C. After 24 hours of incubation 0.2 cc aliquot was withdrawn from the reaction mixture, mixed with 2 cc of 0.01 N iodine-potassium iodide solution, and diluted with water to 25 cc. After standing for 15 minutes, the optical density at 620 mμ of the solution was read in a Pulfrich photometer in a 1-cm cell using the filter S_{61}. The blank value read immediately after the addition of the enzyme was subtracted from the observed value, to obtain the increment of optical density. A good result may be obtained as well by the use of a photoelectric colorimeter, but a Pulfrich photometer was used in the present study, owing to the lack of proper filter in the former instrument.

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[4] G. W. Thomas, W. J. Whelan, and S. Peat, *Biochem. J.*, **47**, xl (1950).

2. Relationships among the Increment of Optical Density, the Reaction Period, and the Amount of Enzyme.

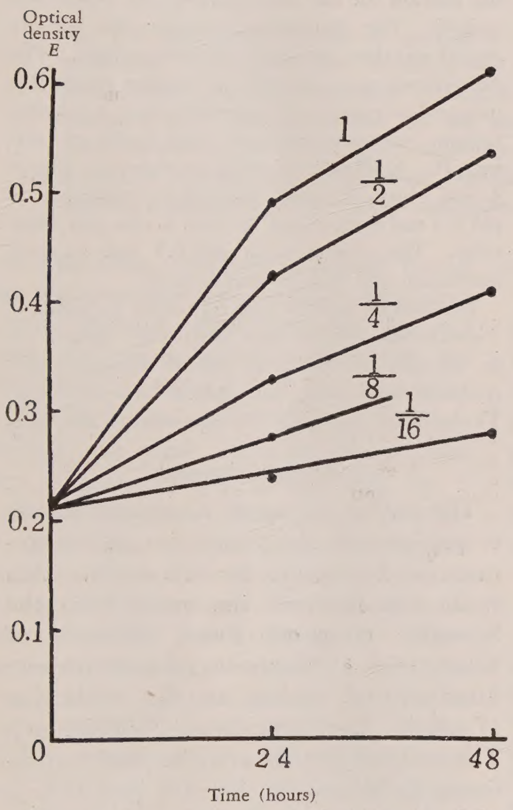

Fig. 1. Increase in the Optical Density of the Starch-iodine Reaction caused by Isoamylase.

A Properly diluted isoamylase solution was added to a mixture of 5 cc 1% solution of glutinous rice starch and 1 cc 0.5 N acetate buffer of pH 6.2. Total volume 7 cc, incubation at 20 C. At various time intervals 0.2 cc aliquot of the reaction mixture was mixed with 2 cc of 0.01 N I_2 solution, and diluted to 25 cc. Optical densities (*E*) of these solutions at 620 mμ were read in a Pulfrich photometer using 1-cm cell.

Figures on the curves represent relative amounts of enzyme used.

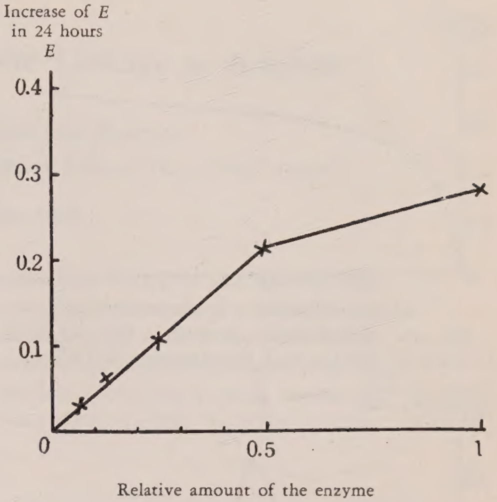

Fig. 2. Relationship between the Amount of Enzyme and the Increment of Optical Density.

(For experimental details see description under Fig. 1.)

When the isoamylase action was followed under the conditions described above, the optical density of the iodine coloration increased linearly with time in the earlier stage of the reaction, and the rate of increase gradually diminished in the later stage. The process of reaction is illustrated in Figure 1, in which the values except at 24 and 48 hours are omitted for brevity. The increase in the optical density was linear up to 24 hours of reaction with relatively low concentrations of the enzyme. Increment after 24 hours was proportional to the amount of enzyme within a limited range, namely, the value up to 0.22 of optical density (Fig. 2). Thus the enzyme activity could be determined within a range, the enzyme solution to be tested being diluted so as to come into this range.

The reaction period could be reduced by increasing the amount of enzyme, and a period of 24 hours was chosen for experimental convenience.

3. Optical Density and the Amount of Iodine.

In order to determine the amount of iodine solution to be added for the measurement of iodine coloration, each 0.2 cc of the same reaction mixture was treated with varying amounts of 0.01 N iodine solution, and a curve, as shown in Figure 3 was obtained. With low concentrations of iodine, the curve showed a sharp slope indicating that considerable errors may be caused

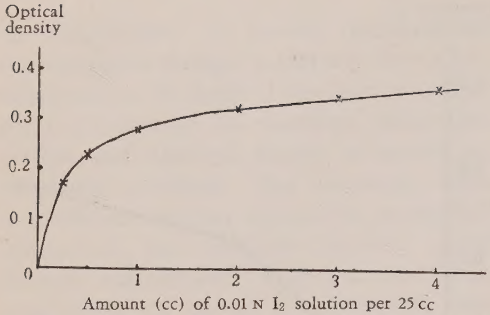

Fig. 3. Relationship between the Optical Density at 620 mμ and the Amount of Iodine.

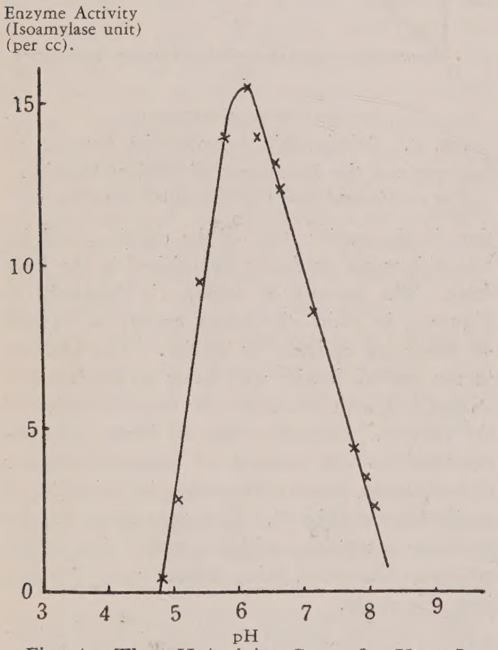

Fig. 4. The pH-Activity Curve for Yeast Isoamylase.

Temperature: 20°C. Buffer: 0.1 N Veronal-Sodium acetate-HCl.

The enzyme activity was expressed in the "isoamylase unit", taking an increase of E 0.200 as 10 units.

by a slight variation in the iodine concentration. In order to minimize these errors, the amount of iodine was decided, according to the results shown in Figure 3, on 2 cc of a 0.01 N solution per 25 cc of the final solution to be measured.

4. pH-Activity Curve of Isoamylase.

The method described above provided a reliable method for the determination of isoamylase activity. The dependence of isoamylase activity on pH was then estimated with the method. The experiments were carried out on yeast isoamylase prepared as previously reported[1]) with a veronal-sodium acetate-hydrochloric acid buffer of varying pH. A pH-activity curve as shown in Figure 4 was obtained, which showed the maximum at pH 6.2 and ateep slopes in both acidic and basic sides. The maximum at pH 6.2 was in good agreement with former description[5]).

The enzyme activity was expressed in terms of "isoamylase unit", thus tentatively defined as an increase of 0.200 in optical density, under specified conditions, corresponded to 10 units. The ordinate in Figure 4 is expressed in this unit.

Acknowledgement

The author expresses his sincere thanks to professor S. Funahashi for encouragement and interest in this work. This study was aided by the grant from the Scientific Research Fund, Ministry of Education. (Composite research subject: Fundamental studies on the utilization of starch. Representative: Zirô Nikuni). The author's thanks are also due to professor Z. Nikuni.

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5) S. Nishimura, *J. Agr. Chem. Soc. Japan*, **6**, 160 (1930).

Absence of β-Glucosidase-Labile Linkage in Amylose

By Akira BABA and Hidejirō KOJIMA
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Received May 25, 1955

The β-glucosidic linkage in amylose as proposed by Peat, Pirt and Whelan[1] was examined. An amylose limit β-dextrin was prepared with sweet potato amylose and sweet potato crystalline β-Amylase. β-Glucosidase of apricot emulsin was purified to eliminate the α-amylase action. In our experiments on the hydrolysis of amylose limit β-dextrin by several enzymes, commercial emulsin containing a small amount of α-amylase attacked the amylose limit β-dextrin, but the crystalline β-amylase and the purified β-glucosidase were found ineffective.

In 1952 Peat, Pirt and Whelan[1] found that the amylose obtained from natural sources (potato, sago, tapioka, maize) were not completely hydrolyzed by crystalline β-amylase, and the extent of conversion to be about 70% as maltose, whereas the same amylose preparations were almost entirely saccharified by a soybean β-amylase. Hence they considered that the soybean amylase should contain a factor attacking an anomalous linkage which arrested the β-amylolysis, and called this factor Z-enzyme. Furthermore, they have concluded that the Z-enzyme is a β-glucosidase because its enzyme action can be simulated by the emulsin. Toward the above opinions of Peat, Pirt and Whelan, obvious evidence by Hopkins and Bird[2] has been given on the existence of the arrest point of β-amylolysis in natural amylose, but suggested that the action considered as that of the Z-enzyme is explainable to the contaminated trace amount of α-amylase. Fukumoto and Tsujisaka[3] also reported the absence of Z-enzyme in soybeans.

By using the sweet potato amylose prepared by the method of Schoch[4] and the sweet potato crystalline β-amylase, we also have confirmed that the arrest point of β-amylolysis is about 70%. In this experiment we have purified the β-glucosidase of apricot seed and observed that the amylose limit β-dextrin was not affected by the β-glucosidase.

Experimental

Purification of β-glucosidase. In our experiment we used the seed of apricot as the β-glucosidase source, from the reason that it was easy to obtain. We have observed that the enzyme prepared by the tannin precipitation method of Helferich and coworkers[5] contained a trace amount of α-amylase. Hence the following procedure was applied. Five hundred grams of dry apricot seeds were groud with 2500 ml. of acetate buffer (pH 4.8, 0.02 M). After filtration, 42.9 ml. of 20% zinc sulfate solution was added per liter of filtrate, and the precipitate of inactive protein separated by filtration and discarded. Solid ammonium sulfate was added to the enzyme solution and brought to 0.25 saturation, and the precipitate filtered off. Solid ammonium sulfate

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[1] S. Peat, S. J. Pirt and W. J. Whelan, *J. Chem. Soc.*, **1952**, 705-733.

[2] R.H. Hopkins and R. Bird, *Nature*, **172**, 492 (1953).

[3] J. Fukumoto and Y. Tsujisaka, *Science and Industry, Japan*, **28**, 282 (1954),

[4] T.J. Schoch, *J. Am. Chem. Soc.*, **64**, 2957 (1942).

[5] B. Helferich, S. Winkler, R. Gootz, O. Peters and E. Günther, *Z. physiol. Chem.*, **208**, 91 (1932).

Table I
Purification of Apricot β-glucosidase.

Step	Volume ml.	Unit		Protein*** mg	Specific activity per mg protein	
		β*-glucosidase	α-** amylase		β-glucosidase	α-amylase
Crude extract	1630	2250	50	14670	0.51	0.003
After $ZnSO_4$ treatment	1560	1870	<9	6240	0.28	<0.001
After $(NH_4)_2SO_4$ fractionation (0.25–0.55 Sat)	55	1760	trace	1430	1.23	
After dialysis	35	1720		860	2.00	
Acetone fractionation (45%)						
After acetone and petroleum ether washing	22	1670		570	2.92	
Alcohol fractionation (25–45%)						
After dialysis	10	1000		160	6.25	

* Determined by the method of Sumner et al[7].
** Determined by the method of Wohlgemuth[8].
*** Determined by the method of Gornal et al [9].

Table II
Action of purified Apricot β-glucosidase on Amylose at pH 4.8, 30°C.

	Absorption value (680 mμ.) Reaction time		
β-glucosidase	1hr	5 hrs	24 hrs
10 unit	0.58	0.55	0.58
5 unit	0.56	0.58	0.58
1 unit	0.56	0.58	0.59
Blank	0.57	0.58	0.58

Digestion mixtures were prepared by the method of Peat, Pirt and Whelan[1]. But crystalline sweet potato amylose was used.

was added up to 0.55 saturation to the filtrate, and the precipitate was centrifuged and then dialyzed in cellophane against distilled water for three days and the insoluble precipitate was centrifuged out. The dialyzed solution was cooled in a refrigerator and cold acetone was added to 4.5 volume %. After centrifugation the precipitate was washed with cold acetone and petroleum ether, and dried in vacuo. The enzyme powder was dissolved into the 20 ml. of acetate buffer (pH 4.8, 0.02 M). To this enzyme solution ice cooled 85 volume % alcohol (containing 1/10 volume of pH 4.8, 0.2 M acetate buffer) was added up to 25 volume % and the precipitate centrifuged and discarded. The alcohol concentration was raised to 45 volume % and after cooling at 0°C. for thirty minutes, the precipitate was collected by centrifuging and dissolved in distilled water. α-Amylase activity was eliminated almost entirely by the above purification procedure as shown in Table 2, however, after testing by Wijsman's method[6] the concentrated enzyme solution indicated the presence of a negligible trace of α-amylase activity.

In the comparable experiment, the commercial emulsin indicated stronger α-amylase activity by the above Wijsman's test. Salicin was used as a substrate in the determination of β-glucosidase activities, but strong cellobiase action was observed by paperchromatography in the digestion of cellobiose.

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[6] Z. Nikuni, "Starch Chemistry" Asakura Press, 1953, p. 407.

Preparation of crystalline β-amylase. Sweet potato β-amylase thrice recrystallized was prepared by the method of Balls, Walden and Thompson[10]. The enzyme indicated a slight trace of α-amylase action in the test of Wijsman[6]. The trace of α-amylase was not eliminated even after the enzyme was treated with acetic acid at pH 3.6 for 2 weeks in an ice chest. We therefore used the thrice recrystallized enzyme for this experiment.

Crystalline malt α-amylase. We used the enzyme kindly sent to us by Walden and it was prepared by the method of Schwimmer and Balls[11]. The enzyme activities were determined by the method of Wohlgemuth[8].

Preparation of amylose limit β-dextrin. The sweet potato amylose was prepared by the method of Schoch[4] and thrice recrystallized as a precipitate of butanol complex. The 300 mg of dried amylose was wetted with petroleum ether (b.p. 30–60°C) and 12.5 ml. of N-sodium hydroxyde solution added. By shaking this mixture in a boiling water bath for a few minutes, amylose dissolved completely. After cooling, the solution was poured into a 500 ml. volumetric flask with a small amount of distilled water, and 0.5 ml. of 0.01% phenolphthalein solution was added as an indicator. The solution was neutralyzed by N-hydrochrolic acid, and 60 ml. of pH 4.8, 0.2 M acetate buffer was added. The flask was filled up with distilled water and placed in a thermostat bath at 30°C. Finally, 2 ml. of crystalline β-amylase suspension in 0.7 saturated ammonium sulfate solution (containing 52800 units, determined by the method of Peat, Pirt and Whelan) was added and covered with a toluene layer. The reducing sugar produced by the digestion was determined by Somogyi's iodometric method[12]. After forty eight hours incubation the conversion of amylose reached a limit at 73.4% as maltose. The digestion mixture was heated to boiling and immediately filtered, and this treatment was repeated over again. The clear and colorless solution of amylose limit β-dextrin resulting from the above treatment was used as the substrate for the experiments shown in Table III. By the above digestion method 3 g. of amylose was converted into amylose limit β-dextrin, and the amylose limit β-dextrin in the digestion mixture separated by the method of Kerr and Cleaveland[13], moreover the DPn. of original amylose and the amylose limit β-dextrin were determined by the method of Potter and Hassid[14], and found to be 865 and 524 respectively.

Preparation of digestion mixtures for the test of the enzyme actions on amylose limit β-dextrin. To examine the action of purified β-glucosidase on amylose limit β-dextrin, 50 ml. of amylose limit β-dextrin solution prepared by the procedure mentioned above was used as the substrate. The solution was placed in the thermostat bath and when the solution temperature reached 30°C. the enzyme solution was added. Volume of the reaction mixture was adjusted to 53 ml. with distilled water, and finally toluene was added. Several enzyme actions on amylose limit β-dextrin were examined at the same time as shown in Table III.

Determination of absorption value. Five ml. of each reaction mixture above was pipetted at intervals for determination of absorption value. The reaction mixture was poured into the iodine solution, prepared with 1ml. of a solution containing 0.1g. iodine and 1g. potassium iodide in 100 ml. water, and 45 ml. of water. The absorption values were read in a logarithmic scale of COLEMAN photoelectric colorimeter fitted with a 14–214 filter and a test tube of 15 mm. in diameter. This reading was set to zero against the diluted iodine solution (instead of the reaction mixture, 5 ml. distilled water was added to the 46 ml. of diluted iodine solution).

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[7] J. B. Sumner and G. F. Somers, "Laboratory Experiment in Biological Chemistry" 1949, p. 144.

[8] J. Wohlgemuth, *Biochem. Z.*, **9**, 1 (1908).

[9] A. G. Gornall, C. J. Bardawill and M. M. David, *J. Biol. Chem.* **177**, 751 (1949).

[10] A. K. Balls, M. K. Walden and R. R. Thompson, *J. Biol. Chem.*, **173**, 9 (1948).

[11] S. Schwimmer and A. K. Balls, *J. Biol. Chem.*, **175**, 465 (1948).

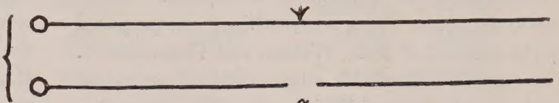
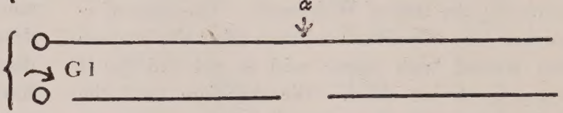
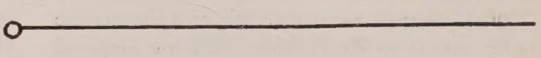
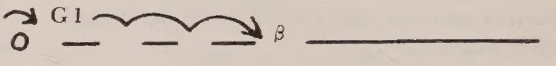
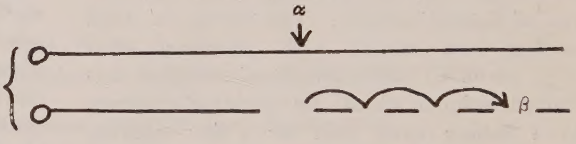
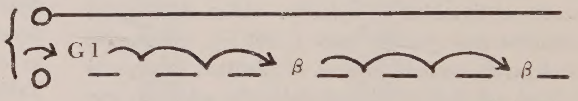
[12] M. Somogyi, *J. Biol. Chem.*, **175**, 19 (1952).

[13] R. W. Kerr and F. C. Cleaveland, *J. Am. Chem. Soc.*, **73**, 2421 (1951).

[14] A. L. Potter and W. Z. Hassid, *J. Am. Chem. Soc.*, **70**, 3774 (1948).

Table III

Action of Purified and Commercial β-glucosidase (emlusin) on Amylose limit β-dextrin

	Absorption value (680 mμ.) Reaction time			Expected action of enzyme(s) (○: hypothetic β-glucosidase-labile linkage)
Enzyme(s)	1hr.	5 hr.	24 hr.	
α	0.37	0.27	0.28	
α+G1	0.35	0.27	0.26	
β	0.38	0.37	0.36	
β+G1	0.37	0.34	0.31	
β+G2	0.15	0.11	0.08	
α+β	0.04	0.02	0.01	
α+β+G1	0.04	0.02	0.01	
Blank	0.41	0.41	0.41	

α: Crystalline malt α-amylase (0.022 unit).
β: Crystalline sweet potato β-amylase (1300 unit).
G1: Purified emulsin (5 unit, specific activity 6.25).
G2: Commercial emulsin (2 unit, specific activity 0.20).

Results

It is remarkable that both crystalline β-amylase (β) and crystalline β-amylase plus purified β-glucosidase (β+G1) indicate just the same faint negligible conversion on amylose limit β-dextrin, compared by the blank test. But other enzymes and enzyme combinations vigorously converted the limit dextrin.

In comparing (β+G1) with (β+G2), commercial emulsin (G2) was found to be able to supplement β-amylase action strongly, but not at all to purified β-glucosidase.

The influence of the purified β-glucosidase on (α+β) amylase action was further examined and found to have no effect.

Discussion

If it should be true that the β-glucosidase-labile linkage exists in one end of the amylose limit β-dextrin molecule, the following assumptions as shown in Table III should be plausible. (1) In comparing with (α) and (α+G1), the decrease of absorption value will be identical. (2) The (β) should not attack the amylose limit β-dextrin. But the (G1) should supplement the action promptly, as described by Peat et al. (3) It will be expected that the (α+β+G1) hydrolyze the amylose limit β-dextrin more rapidly and extensively than the (α+β). However, expectations of (2) and (3) were not satisfactory. Therefore, it seems to be true that the hypothetical "anomalous linkage" arresting the β-amylolysis of amylose can not be a β-glucosidase-labile linkage. On the other hand, comparatively impure commercial emulsin plus β-amylase (β+G2) hydrolyzed the amylose limit β-dextrin considerably rapidly and stronger than the (β+G1). The quantitative estimation of a trace amount of α-amylase and (G1) and (G2) is practically difficult, but we have recognized that the commercial emulsin contains a larger amount of α-amylase than purified β-glucosidase as mentioned above. Therefore, the supplemental effect of (G2) on the β-amylolysis must be due to its contaminated α-amylase action as described by Hopkins et al.

It is a generally well known fact that emulsin contains several enzymes, for example galactosidase and fructosidase etc. In this experiment we have not studied the presence of these enzymes in our purified β-glucosidase and commercial emulsin. Though we could be convinced of the existence of the arrest point in amylose to β-amylolysis, it was also established that the arrest linkage can not be β-glucosidase-labile.

Summary

The β-glucosidase of apricot seeds was purified to a preparation contain a negligible trace amount of α-amylase.

The crystalline β-amylase hydrolyzed amylose to a limit at 73.4% as maltose, so the existence of the arrest point to the β-amylolysis was confirmed.

The purified β-glucosidase could not supplement the crystalline β-amylase, on the hydrolysis of amylose limit β-dextrin.

Hence it may here be considered that the presence of a β-glucosidase-labile linkage in amylose as described by Peat et al., is not convincible.

Acknowledgement

The authors wish to express their gratitude to Dr. Zirō Nikuni and Dr. Kiichi Iwamoto for their interest and helpful suggestions. They are also indebted to Dr. A.K. Balls and Dr. M.K. Walden for furnishing us crystalline malt α-amylase. The expenses of this research has been defrayed by the Department of Education, to which the authors' thanks are due.

Studies on Cycasin, a New Toxic Glycoside, of Cycas revoluta Thunb.

Part II. Hydrolysis of Cycasin with Cycad-Emulsin

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Received May 31, 1955

The enzymatic hydrolysis of cycasin with cycad-emulsin, prepared from the seeds of *Cycas revoluta*, is described. As the complete degradation products we obtained about one mole each of formaldehyde, nitrogen gas, and methanol per mole of cycasin, in addition to glucose, the sugar component. These products are the same as those found in the acid hydrolysis of cycasin which has been reported previously. Therefore, it is concluded that the aglycone of cycasin can not be liberated intact as a single component, but decomposes into those smaller molecules as above even by means of the enzymatic hydrolysis.

In paper I[1] of this series the isolation of cycasin, a new toxic glycoside of *Cycas revoluta*, was reported. The glycoside was identified as glucosyloxyazoxymethane from its chemical properties and absorption spectra. When cycasin was hydrolyzed with acid or alkali, its aglycone was not liberated intact as a single component, undergoing further degradation. For the present purpose of elucidating the behaviors of the aglycone in the enzymatic hydrolysis of cycasin, the decomposition products were examined under various conditions with cycad-emulsin. Since the formation of nitrogen gas from cycasin was proved, it was measured by Warburg's manometer.

Experimental

1. Preparation of Cycad-Emulsin.

Cycad-emulsin was isolated as a dry preparation by Helferich's method[2] as shown in Table I. Containing much insoluble materials, the first acetone powder, Crude Enzyme I, had relatively low activity. Similar treatment was, therefore, repeated again on the soluble part to give Crude Enzyme II. Its activity was standardized[3] with salicin to be 0.402, expressed as β-glucosidase value. The enzyme solution was prepared by macerating a definite amount of this preparation thoroughly with water, centrifuging off any insoluble residue, and making the supernatant up to a volume.

2. Preliminary Experiment.

A mixture of cycasin, enzyme, and buffer solution was taken into a small conical flask, to which an inverted burette was connected. Freshly boiled water was filled in this apparatus, and the flask was submerged in a bath at 45° for 24 hours. Qualitative analyses of the reaction mixture and the gas collected in the burette showed that the enzymatic degradation products were the same as those obtained from the acid hydrolysate of cycasin, that is, formaldehyde, nitrogen gas, methanol and glucose. However, none of the alkali-decomposition products of cycasin, such as cyanide, formic acid, and volatile bases were detected. After several examinations, the conditions, shown in Table II, were proved to be the most appropriate for the manometric estimation of the nitrogen gas

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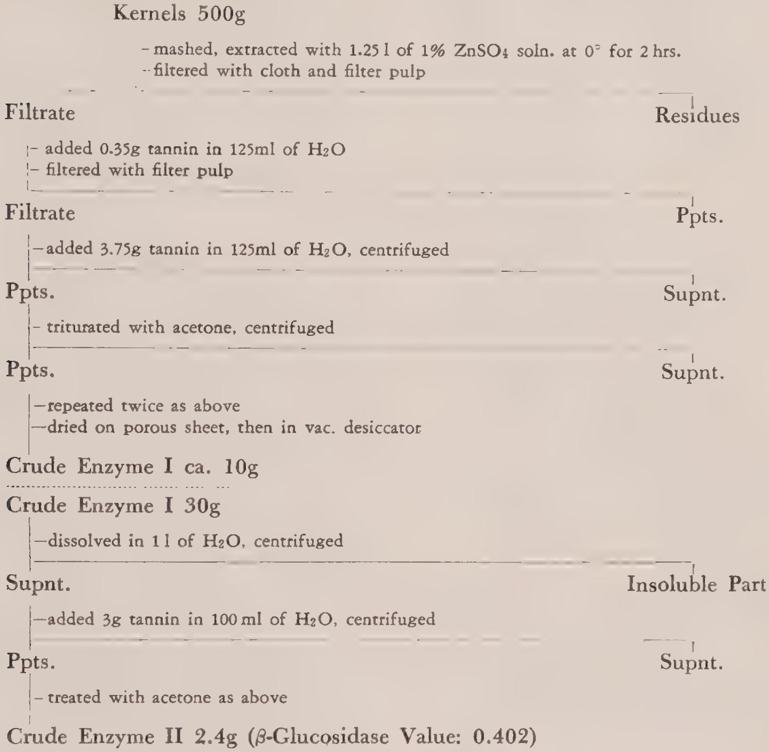
[1] K. Nishida, A. Kobayashi, and T. Nagahama, *This Bull.*, **19**, 77 (1955).

[2] B. Helferich, S. Winkler, R. Gootz, O. Peters, and E. Günther, *Z. physiol. Chem.*, **208**, 91 (1932).

[3] B. Helferich and H. Appel, *ibid.*, **205**, 231 (1932).

Table I

Preparation of Cycad-Emulsin from the Seeds of *Cycas revoluta*



Kernels 500g
- mashed, extracted with 1.25 l of 1% $ZnSO_4$ soln. at 0° for 2 hrs.
- filtered with cloth and filter pulp

Filtrate | Residues
- added 0.35g tannin in 125ml of H_2O
- filtered with filter pulp

Filtrate | Ppts.
- added 3.75g tannin in 125ml of H_2O, centrifuged

Ppts. | Supnt.
- triturated with acetone, centrifuged

Ppts. | Supnt.
- repeated twice as above
- dried on porous sheet, then in vac. desiccator

Crude Enzyme I ca. 10g

Crude Enzyme I 30g
- dissolved in 1 l of H_2O, centrifuged

Supnt. | Insoluble Part
- added 3g tannin in 100 ml of H_2O, centrifuged

Ppts. | Supnt.
- treated with acetone as above

Crude Enzyme II 2.4g (β-Glucosidase Value: 0.402)

Table II

Basal Conditions for Monometric Estimation of Nitrogen Gas Formation

	Quantity Employed	*Location*
M/10 Phosphate Buffer, pH 5.6	1.0 ml	Main Flask
Cycasin soln., 40 μM (10.08 mg) in	0.5 ml	Main Flask
Enzyme Soln., 5 mg in	0.5 ml	Side Arm
20% KOH Soln.,	0.2 ml	Center Well
	Gas Phase, H_2	Temp., 40°

formation. The determinations of the gas as well as formaldehyde produced, were conducted under the same conditions if they are not otherwise described.

3. Amount of Enzyme and Substrate.

The rate of nitrogen gas formation was linear with time after a 30 minutes lag period as shown in Fig. 1, in which the gas formation from a definite amount of cycasin by varying amounts of the enzyme is illustrated. Similar results were also obtained when the substrate concentration was varied. The rate of gas formation after 2 hours, expressed in moles per mole of cycasin, was correlated to the amount of enzyme or substrate as shown in Fig. 2. The rate was almost constant even when the enzyme or substrate concentration was increased. However the total volume of the gas was too small to be measured in the lower concentrations. That is,

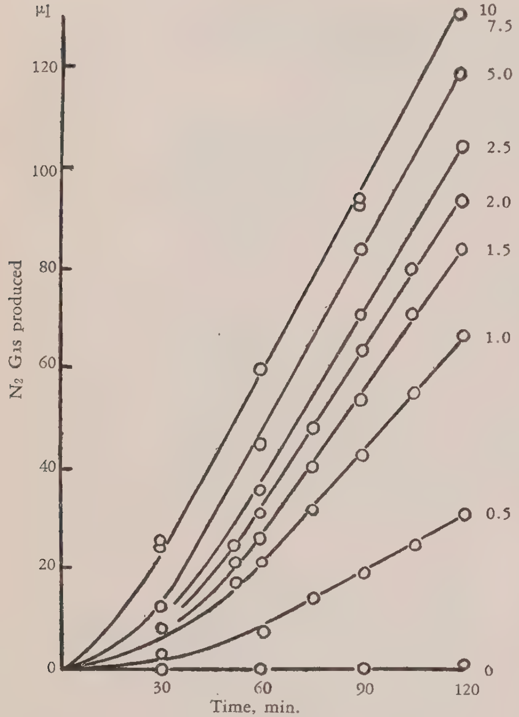


Fig. 1. Formation of Nitrogen Gas from Cycasin with Varying Amounts of Enzyme.

Figures on the right indicate mg of cycad-emulsin used. Other conditions are shown in Table II.

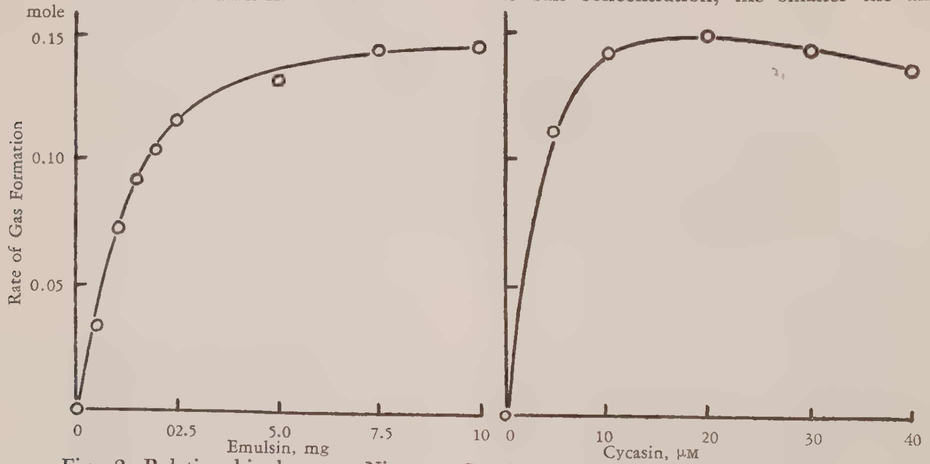


Fig. 2. Relationship between Nitrogen Gas Formation and Amounts of Enzyme or Substrate.

Measured after 2 hrs. The rate of formation is expressed in moles per mole of cycasin.

for example, the gas produced from 10 µM of cycasin with 5 mg of enzyme was only 32 µl even after 2 hours.

4. Influence of Buffer Solution.

Difference in composition of the buffer solution did not influence the gas formation to a considerable degree. Thus, the amount of gas produced was slightly less in acetate and phosphate buffer solution than in citrate-phosphate and citrate-sodium hydroxide buffer of the same concentration.

The nitrogen and formaldehyde formations were studied at varying pH in phosphate-citrate buffer. For the determination of formaldehyde, the reaction was halted after 2 hours by adding 0.5ml of 20% trichloroacetic acid solution into the manometer vessel, and colorimetry with phenylhydrazine[4] was conducted on an aliquot of the supernatant. In a series of experiments in a small conical flask at 50° for 2 hours, formaldehyde formation was proved to be maximum at about pH 5.6. The nitrogen gas formations were also prominent at about pH 5, but any remarkable maximum was not observed. These results are given in Fig. 3.

5. Influence of Emulsin Inhibitors.

In this experiment, 0.5 ml of inhibitor solution was added to the main flask containing 0.5ml of M/5 buffer. When copper sulfate, mercuric chloride or silver nitrate was added, the higher the salt concentration, the smaller the amount

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[4] M. Tanenbaum and C.E. Bricker, *Anal. Chem.*, **23**, 354 (1951).

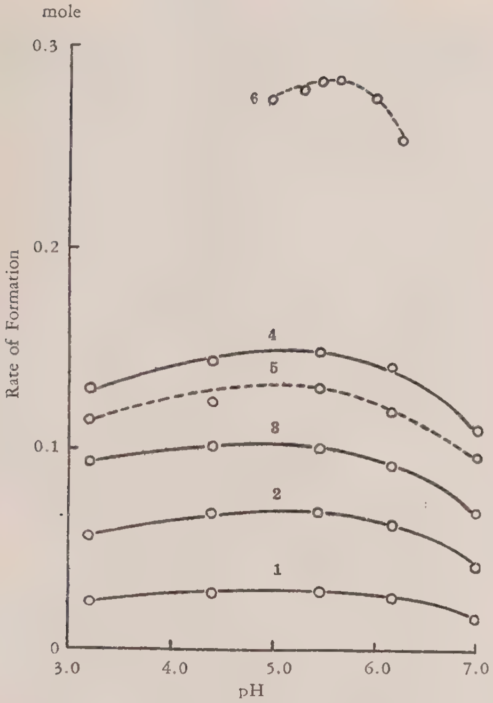

Fig. 3. Influence of pH on Nitrogen Gas and Formaldehyde Formation

Nitrogen gas: at 40° after 30 min. (full line 1), 60 min. (2), 90 min. (3), and 120 min. (4). Formaldehyde: after 120 min. at 40° (broken line 5), and at 50° (6). Buffer: phosphate-citrate. Other conditions are shown in Table II.

of gas produced. Of the salts tested in the same concentration, silver salt was the most inhibitory, and copper salt relatively less effective. (Fig. 4)

Influence of phenylhydrazine, sodium hydrogen sulfite, or formaldehyde, which had been reported as the emulsin inhibitor, was negligible so far as their concentration was low. Formaldehyde was examined for its influence on the gas formation in such a way as Helferich et al.[5] had reported, i.e., an enzyme solution to which less than 0.5% of formaldehyde was added, was employed after being kept at 0° for 24 hours. In this case the influence was somewhat prominent when the concentration was high.

6. Effect of Reaction Temperature.

Since manometric estimation of nitrogen gas was difficult at higher temperatures, the effect of temperature was examined by assaying formaldehyde produced 2 hours after the start of experiment. As shown in Fig. 5, it was observed that with the increase of temperature, the amount of formaldehyde increased and reached to the maximum value of 0.62 moles per mole of cycasin at 65°. However, it turned extremely low at 70°.

7. Complete Degradation.

In order to study the time course of the complete hydrolysis of cycasin, the gas formation was continually measured in an experiment, in which the amount of enzyme was doubled to 10mg and the temperature was raised to 50°. The reaction velocity decreased with the lapse of time as shown in Fig. 6, and after 10 hours the rate of gas formation reached finally 1 mole. Formaldehyde produced was determined on an aliquot of the vessel contents at 2 hour intervals.

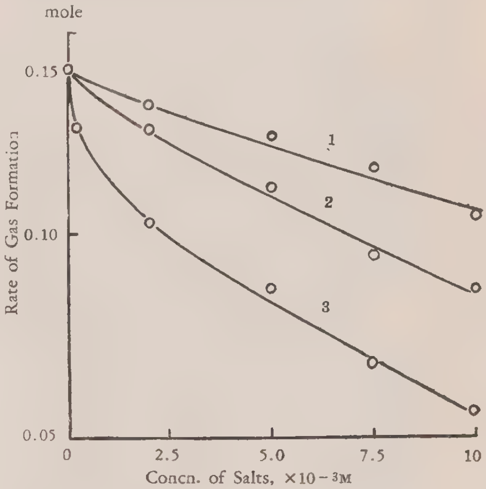

Fig. 4. Influence of Heavy Metal Salts on Nitrogen Gas Formation

1; $CuSO_4$, 2; $HgCl_2$, 3; $AgNO_3$. Measurements were made after 2 hrs. Main flask contained 0.5 ml of the salt soln. and 0.5 ml of M/5 buffer. Other conditions are shown in Table II.

The curve agreed with that of the gas till 90 minutes, but thereafter was gradually slackened and became only about half in the amount of the latter.

The degradation products other than the gas

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[5] B. Helferich and S. Winkler, *Z. physiol. Chem.*, **221**, 98 (1933).

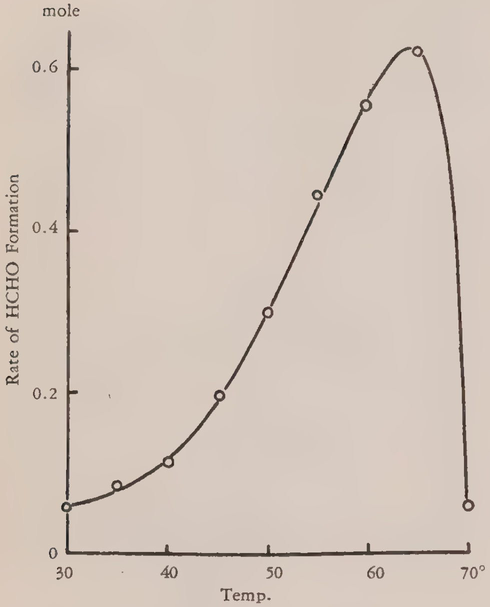

Fig. 5. Formaldehyde Formation after 2 hrs. at Various Temperatures

Other conditions are shown in Table II.

were determined as follows. A mixture of cycasin (400 μM in 5 ml), enzyme (250 mg in 5 ml), and M/10 phosphate buffer solution (10 ml) was incubated at 50° for 20 hours. Two ml of 50% trichloroacetic acid solution was added to the reaction mixture, and each quantitative determination was made on the aliquot of the supernatant. At first 2 ml of the supernatant was steamdistilled after neutralization and the distillate was filled up to 100 ml. The amount of formaldehyde was calculated as 8.83 mg, i.e. 0.74 moles per mole of cycasin.

Since formaldehyde should be removed for the determination of methanol, 10 ml of the supernatant was neutralized, added with 1.5 ml of 10% dimedone solution, and warmed under a reflux for 10 minutes. The filtrate was distilled carefully, and then the colorimetry by usual fuchsine-sulfite method was conducted. It was proved that 1.28 moles of methanol was produced per mole of cycasin.

Furthermore, the sugars in the concentrated supernatant were studied by means of the paper chromatography. The electrolytes of the supernatant had been removed previously with ion exchange resins, Amberlite IR-120 and IR-4B. Since the spot of cycasin[1] could not be found on the chromatogram but only that of glucose was identified, it was assumed that cycasin was completely hydrolyzed. The glucose value found by Hanes method was 0.85 mole per mole of cycasin.

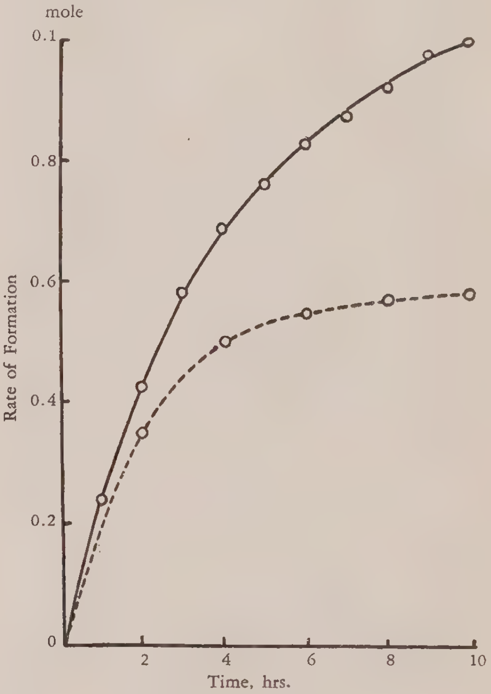

Fig. 6. Complete Degradation of Cycasin into Nitrogen Gas and Formaldehyde

Nitrogen gas, full line; Formaldehyde, broken line. Temp., 50°. Amount of Enzyme employed, 10 mg. Other conditions are shown in Table II.

Discussion

There have been scarcely any report which describes the application of the Warburg's manometric technique for nitrogen gas measurement in biochemical studies. After several preliminary experiments, we could set the basal conditions for manometric determination of the nitrogen gas during the course of enzymatic hydrolysis of cycasin. Untreated juice of the seeds was not suitable as an enzyme preparation, and cycasin which exists in the juice should be removed first, because a considerable amount of gas was produced even in the absence of added substrate. It was also found that for the purpose of this examination, precipitation of the enzyme with tannin, when repeated twice, gave a satisfactory result, and any further purification was unnecessary. By means of exchanging the gas phase of the manometer with hydrogen and adding alkali into the center well, it was possible to make the error due to carbon dioxide negligible, and to measure nitrogen gas exactly.

One of the authors, Nishida,[6, 7] had studied enzymatically the details of cycad-emulsin. According to him, the optimal pH and temperature for the enzyme with the salicin as the substrate were 5.6 and 55° to 60° respectively. In the case of cycasin, too, a remarkable formation of nitrogen gas and formaldehyde was observed at these optimal conditions. The reason that the formaldehyde formation was extremely small in amount at higher temperatures above 65° could be attributed to the heat inactivation of the enzyme.

In the case of the usual enzymatic hydrolysis of a glycoside, the residual glycoside, liberated aglycone or sugar may be determined. The determination of cycasin itself is now being studied, and no appropriate simple method is yet applicable for this compound. On the other hand, the determination of glucose content is also very difficult, because the usual reductometry can not be employed due to the simultaneous formation of formaldehyde. Furthermore, cycasin, being labile in alkali, shows reducing powers by the usual reagents for sugars, and the amount for free glucose will be overestimated.

Further study would be required on these points, but in the experiments described above it is proved that formaldehyde, nitrogen gas, and methanol together with glucose are produced as a result of action of cycad-emulsin on cycasin. Moreover, every degradation product of the enzymatic hydrolysis is obtained in a yield of about 1 mole each per mole of cycasin. The same is true in the acid hydrolysis as well. Therefore it is concluded that when the glycosidic linkage is splitted by enzyme, the aglycone of cycasin can not be obtained as a one component, but is decomposed into those low molecular compounds as shown in the following equation.

$$\underset{\text{Cycasin}}{C_6H_{11}O_5\cdot OCH_2\cdot N(O){:}N\cdot CH_3}+H_2O$$

$$\rightarrow \underset{\text{Glucose}}{C_6H_{12}O_6}+HCHO+N_2+CH_3OH$$

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[6] K. Nishida and A. Yamada, *J. Agr. Chem. Soc. Japan*, **11**, 357 (1935).

[7] K. Nishida, *ibid.*, 1089

Biochemical Studies of Piricularia oryzae

Part III. Formation of Dimethylpyruvic Acid from DL-*Valine*

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Received June 30, 1955

It has been shown that *Piricularia oryzae* could grow in the presence of amino acid, even in the absence of a proper carbon source and that the first step of utilization was the formation of the corresponding α-keto acid by deamination in the medium. For further confirmation of this process, DL-valine was used as the amino acid to be tested in the current experiment. In each of the three cases, that is, DL-valine alone, DL-valine plus arsenite and DL-valine plus sucrose, the dimethlpyruvic acid formed was identified as its 2, 4-dinitrophenylhydrazone. Even in the presence of a sufficient amount of sucrose, some parts of DL-valine added were found to be utilized as a carbon source through the conversion to its α-keto analog.

It has been shown by Otani[1] and Tanaka et al[2] that rice blast disease is likely to break out in a high probability during the growth-period of the rice-plant containing in its body a comparatively high amount of water-soluble nitrogen compounds including acidic amino acids, their amides and other amino acids. They have also shown that in any growth-period these nitrogen compounds are found in a far greater amount in host plants sensitive to blast disease than in those which are resistant. These findings seem to require further research on the attitude of *P. oryzae*, the pathogenic fungus causing blast disease, toward the biochemical condition of the host plant described above. The present authors have already found[3] that this fungus has the ability to utilize amino acids as a good carbon source and to form the corresponding α-keto acids by deamination as the first step of its utilization; thus α-keto *iso*-caproic acid has been proved from L-leucine by the authors. For further confirmation of this utilization process, the present communication records the formation of dimethylpyruvic acid from DL-valine in a medium containing both sucrose and amino acid as well as in media containing either amino acid alone or amino acid and arsenite respectively.

Experimental and Results

P. oryzae was grown at 27–8°C in 30 ml. of a medium containing KNO_3, 60 mg; $CaCl_2 \cdot 2H_2O$, 3 mg; $MgSO_4 \cdot 7H_2O$, 15 mg; KH_2PO_4, 30 mg; $MnSO_4 \cdot 4H_2O$, 0.05 mg; $ZnSO_4 \cdot 7H_2O$, 0.4 mg; $FeSO_4 \cdot 7H_2O$, 0.2 mg; thiamine, 10γ; biotin, 1γ; sucrose, 900 mg. After 14 days of incubation the mycelium of the three culture flasks were transferred together, as previously described[3], to 90 ml. of the second culture medium and further incubation was made at 27–8°C. Of this second culture medium, No. 1, contained three times as much the amount of each material as in the first medium and also 150 mg of DL-valine.; No. 2, only DL-valine 150 mg, in place of sucrose; No. 3, only DL-valine 150 mg, and potassium arsenite, 36 mg. Active growth was observed in both No.

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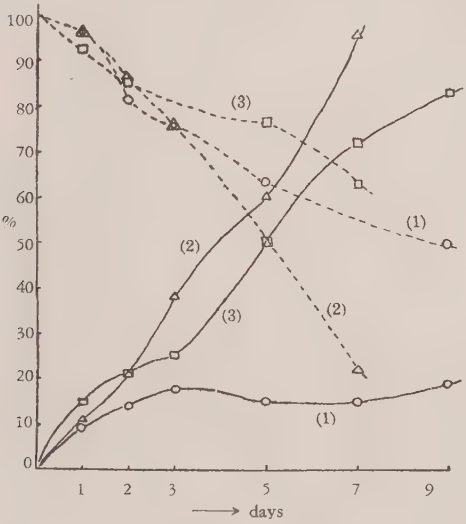
[1] Y. Otani, Memoirs Fac. Agr., Hokkaido Univ., **1**, 375, (1952).

[2] S. Tanaka and F. Katsuki, *J. Chem. Soc. Japan*, **73**, 303 (1952).

[3] Y. Nakamura and T. Shimomura, *J. Agr. Chem. Soc. Japan*, **27**, 694 (1953).

1 and No. 2. After three days, white aerial mycelium was observed, which covered over the liquid surface of the flask within seven days. On the other hand, in No. 3 with arsenite, almost no aerial mycelium was formed and growth appeared to cease within three days.

The ammonia- and amino-nitrogen contents of each flask were measured at appropriate intervals by usual methods. The results obtained are given in the figure. In the three cases, the velocity of ammonia formation and the amount of ammonia production were the largest in No. 2, followed by No. 3. In No. 1 the amount of ammonia produced was found to be 17% three days later, followed by almost no further increase within the succesive nine days. These results are in good accord with those obtained in the experiment, when L-leucine was used as a carbon source3). In each case, the decrease of amino acid by deamination was found to be almost identical in amount up to the first three days but further incubation revealed that the largest decrease of amino acid followed in No. 2, this corresponding to the increase of ammonia.



Ammonia-and Amino-Nitrogens from DL-Valine by *P. oryzae*.

(1) DL-valine+sucrose; (2) DL-valine only; (3) DL-valine+arsenite.

—— represents ammonia-N, and ---- amino- N. Values are expressed as percentage to the DL-valine added.

In spite of the smaller formation of ammonia in No. 1, a relatively larger decrease of amino acid was observed. This fact appears to indicate the occurrence of absorption and utilization of some parts of the amino acid added as a direct nitrogen source, particularly seen in the later stage of incubation. This interpretation seems to be supported by the fact that the total sum of ammonia- and amino nitrogen amounted to 95–96% of the theoretical value during the first three days of incubation but after five days it reduced to 77% and after nine days to 68%. In this case, however, it must be recognized that certain parts of DL-valine added were utilized as a carbon source, especially in the early stage of incubation. This can also be confirmed by the identification of keto acid from DL-valine as described below. It has been learned that even in the presence of sucrose as a carbon source amino acid acted, not only as a nitrogen source, as has been shown by Leaver et al,4) and Otani,5) but also as a carbon source accompanying the deamination process in the medium itself. The occurrence of such an utilization process may be suggested from Eades' report6) that the activity of the keto analog of valine is only 50% of this amino acid toward microorganisms (lactobacillus).

In No. 3, arsenite added, a good correspondence was found between the increase of ammonia- and the decrease of amino-nitrogen during the early stage of incubation. Thereafter, considerable deviation was observed in both nitrogen forms; the decrease of amino nitrogen slowed down and the increase of ammonia became yet more vivid. Taking account of hardly any recognizable growth in this case, the only one possible explanation of this fact is that it should be attributed to partial autolysis caused by the inhibition in the metabolic process, due to arsenite. Among the three cases, the highest accumulation of α-keto acid was qualititatively recognized by the addition of 2,4-dinitrophenylhydrazine to the culture medium. In conformity to the experimental data, and the discussion above, which suggest the formation of α-keto

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4) F.W. Leaver, J. Leal and C.R. Brewer, *J. Bact.*, 54,401 (1947).

5) Y. Otani, *Ann. Phytopath. Soc. Japan*, **17**, 9 (1952).

6) C.H. Eades, Jr., *J. Biol. Chem.*, **187**, 147 (1950).

acid, namely dimethylpyruvic acid, both the isolation and identification of this acid mentioned above, were carried out here in the three cases respectively.

About 90 ml of culture solution (No. 3) was separated from the mycelium after seven to eight days' incubation and concentrated under diminished pressure at a range of pH 8.0–8.1 to a small volume, ca. 2–3 ml, then acidified with 2 ml of 5% sulfuric acid and reconcentrated at 60°–65°C under 10 mm. Hg. This distillation process was repeated thrice and the total distillate was treated with 2,4-dinitrophenylhydrazine solution in 2N-HCl. A yellow crystal mass was isolated, which melted at 191–2°C after being washed with water. After repeated recrystallization from acidic ethanol, by adding water without heating, yellow needles were obtained, which melted at 195–6°C (Found: C, 44.13; H, 3.95. Calcd for $C_{11}H_{12}O_6N_4$: C, 44.59; H, 4.05). This melting point was almost identical with that described by Krebs[8] and Meister.[8] Yield, ca. 12 mg as 2,4-dinitrophenylhydrazone. Application of the same operation to No. 2 gave a yellow crystal of 2,4-dinitrophenylhydrazone, 5 mg, which melted at 195° and gave a mixed melting point at 196°C for that obtained from No. 3 (no temperature depression). The identification of this acid in No. 1 was pretty difficult, owing to its low production and also further utilization, but the addition of arsenite to No. 1 brougth the same effect to the formation and accumulation of this acid as observed in No. 3. By application of acidic ether extraction or distillation keto acid was recognized as the same hydrazone which gave a mixed melting point of 196°C in the crystals described above (Found: C, 44.78; H, 4.10). These results indicate the possibility of α-keto acid formation from DL-valine in both the absence and presence of a carbon source.

Further experiments were conducted on α-amino-*n*-butyric acid and phenylalanine as a carbon source. The formation of phenylpyruvic acid from the latter and an unidentified carbonyl compound from the former (its 2,4-dinitrophenylhydrazone, m.p. 153–4°; C, 46.86; H, 5.02; $R_F=0.91$ to *n*-butanol saturated with 3% ammonia) will be described in our next report.

Summary

P. oryzae is able to grow by its utilization of DL-valine in the absence of a proper carbon source. The formation of α-keto acid, corresponding to the amino acid mentioned, dimethylpyruvic acid is the first step of utilization. This acid (dimethylpyruvic acid) was identified as its 2,4-dinitrophenylhydrazone. A comparatively small amount of this keto acid could be recognized even in the culture medium containing both DL-valine and sucrose. This fact appears to indicate that certain parts of DL-valine, when added to a medium containing sucrose sufficient for growth, were utilized not as a nitrogen source but as a carbon source, pesecially in the early stage of incubation.

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7) H.A. Krebs, *Z. Physiol Chem.*, **217**, 191 (1933).
8) A. Meister, *J. Biol. Chem.*, **197**, 309 (1932).

Blasticidin, A New Anti-Phytopathogenic Fungal Substance. Part I.

By Kazuo FUKUNAGA, Tomomasa MISATO, Itaru ISHII and Masaru ASAKAWA
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Received July 15, 1955

A new antifungal substance, designated blasticidin by the authors, was isolated from the culture broth of a species of *Streptomyces*. This antibiotic is particularly effective against rice blast. In this paper a report is made on the taxonomic study of this strain, its cultural condition, the purification process and some properties of this antibiotic.

In recent years much attention has been given to the application of antibiotics for the control of blast, which is the most destructive disease of rice plants in Japan. Cephalothecin[1] and antiblastin[2] have been reported for this purpose.

Previously the authors have obtained several effective strains against *Piricularia oryzae in vitro* and *in vivo*.[3] The strain 2A–327, one of them, kindly presented by the Antibiotic Research Group, Dept. of Agricultural Chemistry, Faculty of Agriculture, Univ. of Tokyo with whom the authors are cooperating, from their culture collection, which was isolated from the soil at Gazaki, Wakayama Prefecture, was found to belong to a species previously undescribed. Hence it was named *Streptomyces griseochromogenes* Fukunaga. Furthermore, the active substances produced by this strain were named "Blasticidin A, B and C", since they were recognized to be new antibiotics, in respect of their unique properties.

Taxonomic Study of the Strain

Morphological and biochemical characteristics of the strain 2A–327 on various media were observed by Waksman's[4] and Krainsky's[5] method, and in addition, Pridham's[6] utilization test of carbon source by this strain was examined.

This strain belongs to the so-called chromogenic type. Among known species, *Streptomyces resistomycificus*,[7] *S. mirabilis*,[7] *S. flavochromogenes*,[8] *S. olivochromogenus*[8] and *S. diastatochromogenes*[8] somewhat resemble this strain. The descriptions of this strain are summarized in Table I and II, in comparison with *S. diastatochromogenes*, which had similarities in more points than the others, and the oligomycin-producing strain, recognized as a *S. diastatochromogenes*-like strain by M. S. Robert.[9]

Streptomyces resistomycificus differs from the strain 2A–327 in the color of its aerial mycelium observed on various media, and also in the point that it produces an aerial mycelium on nutrient agar and a soluble pigment in glucose-asparagine agar. *S. mirabilis* has a different form of aerial mycelium and a different optimum temperature from this strain. *S. flavochromogenes* produces an aerial mycelium on nutrient agar and a grayish soluble pigment in this, whereas the strain 2A–327 does not produce any aerial mycelium but

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[1] H. Yoshii, *Ann. Phytopathol. Soc. Japan*, **13**, 37–40 (1949).

[2] H. Suzuki, *Ann. Phytopathol. Soc. Japan*, **18**, 138–139 (1954).

[3] K. Fukunaga et al, *Ann. phytopathol. Soc. Japan*, in press.

[4] S. A. Waksmann, *Soil Soc.* **8**, 71 (1919).

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[8] Bergey s Manual of Determinative Bacteriology, 6th ed., (1948).

[9] M. S. Robert, H. P. William & M. Elizabeth, *Antib. & Chemo.* **4**, 962 (1954).

Table I

The Biological Characteristics of the Strain 2A–327, *Streptomyces diastatochromogenes* and Oligomycin-producing Strain

Medium	Strain 2A–327	*St. diastatochromogenes*	Oligomycin-producing strain
Czapek's agar	Growth: spreading, thick, with reverse Orange Cinnamon (XXIX 13″) A.m.: white, on Light Neutral Gray (L III) S.p.: none		Abundant Pallied Mouse Gray aerial mycelium; reverse of Culture Antique Brown
Calcium malate-glycerin agar	Growth: good, reverse of culture Mustard Yellow (XVI 6) A.m.: abundant, white, or Light Neutral Gray S.p.: none	Medium sized colonies, colorless with gray aerial mycelium	Abundant Pallied Mouse Gray aerial mycelium; reverse of culture Cartridge Buff; no diffusible pigment
Glucose-asparagin agar	Growth: restricted, Ivory Yellow (XXX 21″ f), penetrating into the media A.m.: none, later white (21 days) S.p.: none	Same as on Ca-maltate agar	Sparse, white aerial mycelium; reverse of culture Ivory Yellow; no diffusible pigment
Nutrient agar	Growth: restricted, opalescent A.m.: none S.p.: brown	(Peptone agar 0.5%) Medium sized colony, with white, later light gray aerial mycelium; brown pigment	(Glucose-peptone agar) Abundant Pale Gull Gray aerial mycelium; reverse of culture Buckthorn Brown; no diffusible pigment
Starch agar	Growth: abundant, reverse of culture Ivory Yellow A.m.: Powdery, white, or Mouse Gray (LI 15′′′′) S.p.: none Starch hydrolysis on starch plate: 3–4 m.m. wide (7 days) 9–12 m.m. wide (14 days)	Same as on Ca-maltate agar; weakly diastatic	Abundant Pale Quaker Drab aerial mycelium; reverse of culture Cinnamon; diffusible pigment Snuff Brown; starch digested
Potato plug	Growth: abundant, wrinkled, Snuff Brown, (XXIX 15″k) A.m.: white, Mouse Gray Color of plug: dark brown to black around growth	Light gray colonies; gray aerial mycelium; medium; colored black	Moderate Carbon Gray aerial mycelium, Raw Umber diffusible pigment
Carrot plug	Growth: none Color of plug: unchanged		
Gelatin 15–18°C.	Growth: wrinkled, on liquefied portion, yellowish A.m.: white, scant Liquefaction: medium S.p.: dark brown to black	Light gray-colored: Liquefied Soluble brown pigment	(incubation at 19°C.) Growth light; slight liquefaction; Argus Brown diffusible pigment; yellowish flaky sediment
Synthetic solution	Growth: white or yellowish flakes through media A.m.: none S.p.: none		

Medium	Strain 2A-327	*St. diastatochromogenes*	Oligomycin-producing strain
Milk 37°C.	Growth (25°C.): surface ring of Argus Brown (III 13 m), or Bone Brown (XL 13‴m) Coagulation: none Hydrolysis: 8 days at 37°C., while not completed in 21 days. Change of reaction: unchanged (B.C.P. milk) S.p.: brown in hydrolyzed portion		(Litmus milk) Brick red growth ring, buff pink surface growth; soft curd formed and slightly digested; clear straw-colored serum expressed; alkaline reaction
Glucose broth	Growth: colorless flocks on bottom of tube S.p.: brown	Flaky colonies in depth at first, also appearing over the surface later	(Nutrient broth) Growth started on surface, but after two or three days it sank to the bottom of tube where it remained in the form of rather large flocks; the broth remained clear but developed a dark brown diffusible pigment
Tyrosine agar	Growth: orange color A.m.: none S.p.: none	Tyrosinase formed	
Growth on cellulose	none	No growth	(Cellulose agar) Moderate growth aparent after three days incubation; aerial mycelium white; no diffusible pigment
Nitrite formation	Trace (sucrose solution)		
Invertase production	Positive (sucrose solution)	Negative	
Opt. temp.	26–30°C.	35°C.	
Microscopic observation	Spirals: numerous closed spirals as branches of straight hyphae on starch agar: none or curling tips formed on Czapek's agar, glucose-asparagin agar and malate-glycerin agar Conidia: spherical or oval about 1.0–1.5μ	Conidia spherical or oval, about 1.2μ	Condiophores arise from branches of the aerial mycelium and constrict to form oval spores; the conidiophores are straight, hooked, or loose spirals

Table II

Utilization of Carbon Source by the Strain 2A-327

L-Xylose	+	D-Fructose	++	Lactose	++	D-Sorbitol	−	Na-acetate	−
L-Arabinose	+	D-Galactose	++	Raffinose	++	Dulcitol	−	Na-citrate	−
Rhammnose	−	Sucrose	++	Inulin	−	DL-Inositol	++	Na-succinate	−
Glucose	++	Maltose	++	D-Mannitol	++	Salicin	−	Control	−

Base medium: Pridham's medium, ++: good growth and positive utilization; +: moderately good growth and positive utilization —: no growth, no utilization.

Table III

Antimicrobial Spectrum of the Strain 2A-327

Test organisms	Inhibitory diameters(mm)	Test organisms	Inhibitory diameters(mm)
Myc. tuberculosis 607	14.0	*Torula utilis*	13.5
E. coli	0	*Zyg. sulsus*	15.5
B. subtilis	12.0	*Candida albicans*	0
Staph. aureus 209P	0		
Staph. coli	0	*Pricularia Oryzae*	60.0
Pseud. fluorescence	33.0	*Ophiobolus Miyabeanus*	17.0
Asp. oryzae	+	*Gibberella Saubinetii*	18.0
Pen. chrysogenum Q.176	24.3	*Alternaria Kikuchiana*	20.0
Asp. nigar	21.0	*Ceratostomella fimbriata*	30.0
Rhizopus javanicus	14.0	*Glomerella cingulata*	24.0
Sacch. cerevisiae	21.0	*Fusarium Lini*	0

The antimicrobial activity was tested by the agar disc diffusion method.

only a brown soluble pigment. *S. olivochromogenus* differs from this strain in the point that it assumes a dark brown or black color of growth and shows an alkaline reaction in milk medium. As shown in Table I, *S. diastatochromogenes* is quite similar to this strain in the appearance of its growth on several media, but differs from this in the point that it produces a white or gray aerial mycelium on nutrient agar, also by producing tyrosinase in place of invertase. The Oligomycin-producing strain produces a soluble pigment which has a snuff brown color on starch agar, and shows an alkaline reaction in a milk medium, while this strain does not produce a soluble pigment on starch agar, and no change of reaction in the milk medium occurs.

As described above, the strain 2A-327 was found not to be identical with any other species of *Streptomyces* in its cultural characteristics, and after being recognized to be a new species, was thus named *Streptomyces griseochromogenes* Fukunaga.

Antimicrobial Spectrum of the Strain

The antimicrobial activity against 21 kinds of testing organisms was examined by the agar disc diffusion method. As shown in Table III, the antibiotic has been found to be active against various fungi and yeasts, but almost inactive against bacteria.

Assay of Blasticidin

As a correlation has been found between the growth inhibition of *P. oryzae* and *Pen. chrysogenum* Q.176, the latter being used as the testing organism of antifungal activity in the course procedures of fermentation, extraction and purification of blasticidin. After the cultivation of *Pen. chrysogenum* Q.176 for 42 hours at 28°C on the potato glucose agar (agar 2%, pH 5.5), the diameter of the inhibition zone was then measured. As shown in Fig. 1, it was found that a linear relation between logarithm of potency and diameter of inhibition zone consist in a range of 20 to 200 unit/ml in both the cup and paper disc methods. Hitherto, as blasticidin has not been obtained in a crystalline form, 5 mcg of blasticidin A per ml

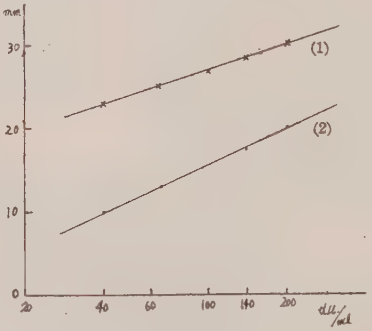


Fig. 1. Relation between the Concentration of Blasticidin A and the Diameter of Inhibition Zone by Cup and Paper Disc Methods.

(1) Cup method
(2) Paper method
(3) d.u. ---- 1 dilution unit = 5 mcg/ml of crude Blasticidin A

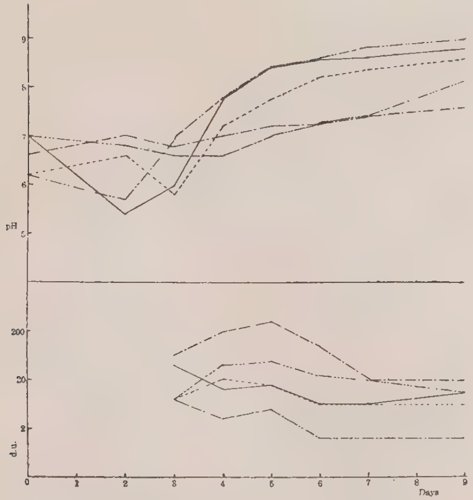


Fig. 2. Difference of Potency in five Media

	Glycerin meat extract medium. glycerin 1.0%, meat ex. 0.5%, NaCl 0.5%.
------ --	Soybean medium. glucose 2.0%, soybean meal 1.0%, KCl 0.4%, yeast (Ebios) 0.02%, meat ex. 0.1%, $(NH_4)_2SO_4$ 0.5%, K_2HPO_4 0.02%, $CaCO_3$ 0.4%.
— ·-·· —	Czapek's medium. sucrose 2.5%, K_2HPO_4 0.1%, $NaNO_3$ 0.2%, KCl 0.05%, $MgSO_4$ $7H_2O$·0.05%.
—··—··—	Modified Waksman's medium. glucose 1.0%, peptone 0.5%, meat ex. 0.5%, NaCl 0.5%, soybean meal 1.0%, K_2HPO_4 0.1%.
—···—···	Yeast medium, glucose 2.0%, $(NH_4)_2SO_4$ 0.5%, yeast 0.25%, K_2HPO_4 0.02%, KCl 0.4%, $CaCO_3$ 0.8%.

i.e, the minimum growth inhibitory concentration against *P. oryzae* by the agar streak dilution method, was adopted as one Dilution Unit.

Cultural Condition for Production

Cultural conditions were examined in shaking culture at 28°C by using a reciprocal shaking machine (120 strokes per minute, 8 cm amplitude). One hundred and fifty milliliters of various culture media were distributed into each 500 ml shaking flask. The change of pH and antifungal potency in the course of fermentation are shown in Fig. 2. The highest concentration of the antibiotic was observed in Czapek's medium. Czapek's medium was therefore adopted as the basal medium. Nitrogen-sources examined were as follows: potassium nitrate, sodium nitrate, ammonium sulfate, ammonium nitrate, ammonium phosphate dibasic, ammonium tartrate, asparagine

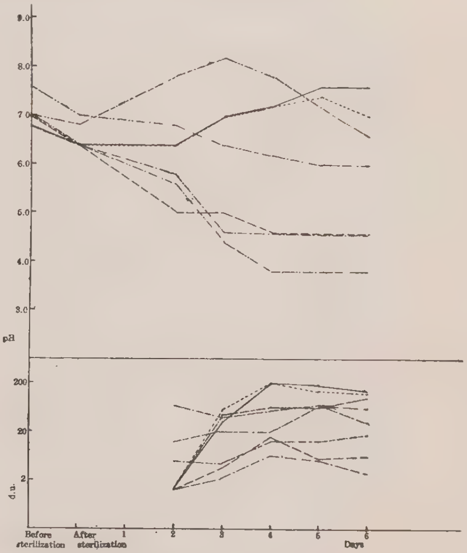


Fig. 3. Effect of Nitrogen-source

	$NaNO_3$	0.2%
----- --	KNO_3	0.2
— — — —	$(NH_4)_2SO_4$	0.2
— · — · — ·	NH_4NO_3	0.2
—··—··—··	$(NH_4)_2HPO_4$	0.2
— · — ·· — ·	Ammonium tartrate	0.2
—···—···—	Asparagine	0.2
—— · —— ·	Peptone	0.2

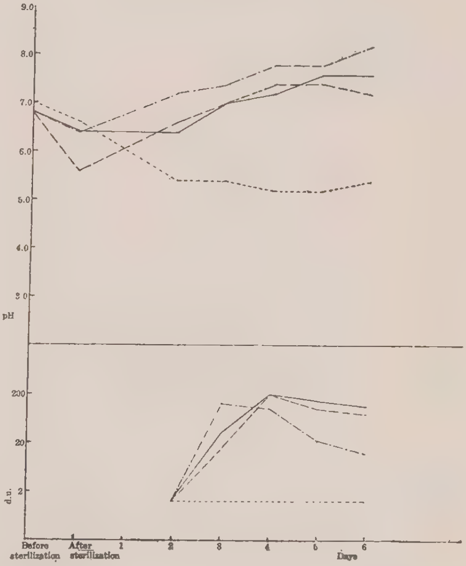
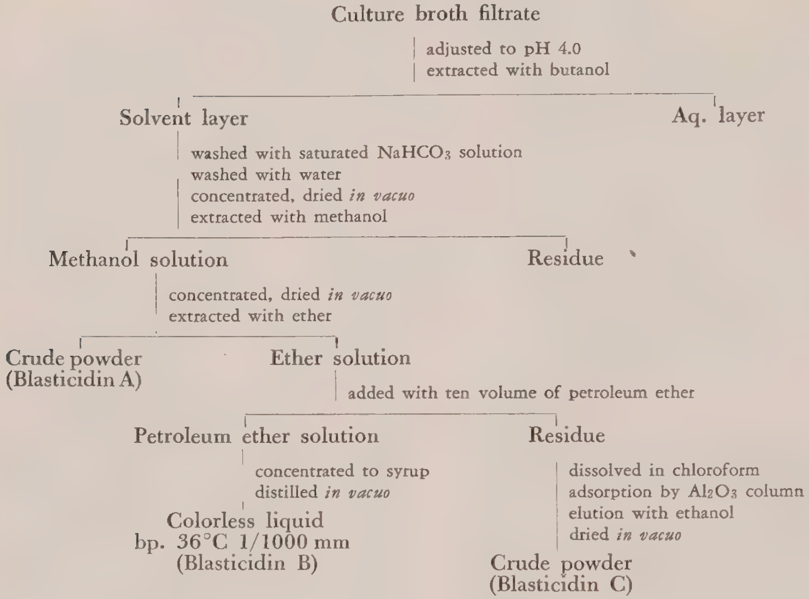


Fig. 4. Effect of Carbon-source

—— sucrose	3%	— — — — glycerin	3%
------ -- glucose	3	—·—··— starch	3

Table IV. Extraction and Purification



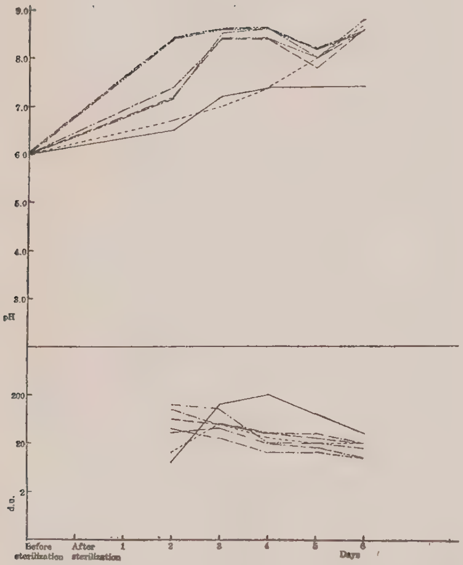


Fig. 5. Effect of the Addition of Some Other Nitrogen Source

and peptone. As indicated in Fig. 3, sodium nitrate and potassium nitrate were found to be most effective. Sucrose was found to be better than glucose, glycerol and starch, as a carbon-source, as shown in Fig. 4. As for other nitrogen sources, the addition of peptone, dry yeast and meat extract were tested. The best result most satisfactory was obtained in the basal medium as shown in Fig. 5. From these results, Czapek's medium was used as the shaking culture medium for this strain. The pertinent initial pH of the medium was 6.0.

Extraction and Purification

The active substance was completely adsorbed by carbon at pH 2 and at pH 8 from the culture broth, and a small quantity by alumina, silica and diatomaceous earth at pH 2. However, the elution of the active substance from carbon was difficult. The active substance was extracted with butanol at pH 2 and at pH 8 from the culture filtrate, and a small quantity of this with butyl acetate and ethyl acetate at pH 2, but neither with benzene, petroleum ether, ethyl ether, chloroform nor carbon tetrachloride. The activity of the culture filtrate was stable when

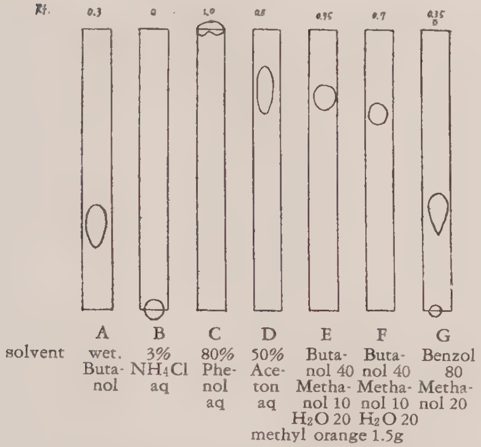


Fig. 6. Summarized Paper Chromatogram
(1) Filter Paper : Toyo No. 131, 1×16 cm
(2) Sample : Culture filtrate broth

boiled at the range of pH 5.0–7.0 for fifty five minutes, but found to be unstable when the pH was lower than 5.0 or higher than 7.0.

Therefore, the purification procedure was carried out by the following method. The five days' cultured broth (pH about 7.2) was first filtrated, the filtrate adjusted to pH 4.0, and the active substance then extracted with butanol. After being washed with saturated sodium bicarbonate aq. solution, next with water, the solvent layer was concentrated and dried *in vacuo*. The residue was dissolved in methanol in order to remove its insoluble impurities, and the methanol evaporated *in vacuo*. A powder thus obtained, was extracted with ether and divided in ether soluble and insoluble parts. After following the procedure shown in Table IV the three active fractions were obtained.

Table V. Antimicrobial Spectrum of Blasticidin

Test organisms	Culture broth filtrate		Blasticidin A		Blasticidin B		Blasticidin C	
	days	M.I.C.	days	M.I.C. (mcg/ml)	days	M.I.C. (mcg/ml)	days	M.I.C. (mcg/ml)
Piricularia Oryzae	3	×100	3	5	3	20	3	25
Ophiobolus Miyabeanus			3	20	3	>85	3	100
Alternaria kikuchiana			3	10	3	>85	3	50
Gibberella Fujikuroi			3	100	3	>85	3	100
Fusarium Lini			3	>100	3	85	3	100
Glomerella Cingulata			3	10	3	28	3	25
Cercospora Oryzae			3	10	3	40	3	100
Ceratostomella fimbriata			3	10	3	40	3	50
Corticium Sasakii			3	10	3	40	5	25
Colletotrichum lagenarium			3	50	3	>40	3	100
Colletotrichum Lindemuthianum			3	10	3	40	5	10
Fusarium oxysporum			3	20	3	>40	3	>100
Gibberella Saubinetii			3	10	3	>40	3	25
Macrosporium bataticola			3	10	3	>40	3	50
Helicobasidium Mompa			5	10	7	40	5	10
Elsinoe fawcettii			5	10	7	40	5	25
Penicillium chrysogenum Q.176	3	×70	3	10	3	>100	3	100
Penicillium glaucum			3	>100	3	>40	3	>100
Aspergillus clavatus			3	10	3	>40	3	100
Aspergillus oryzae			3	>100	3	>40	3	>100
Rhizopus javanicus			3	50	3	>40	3	100
Rhizopus nigricans			3	>100	3	>40	3	>100
Aspergillus niger			3	20	3	>40	3	>100
Aspergillus glaucus			3	10	3	40	3	100

M.I.C. ······· Minimal Inhibitory Concentration

Properties

The summarized paper chromatogram of the culture filtrate broth is shown in Fig. 6. Fraction A is a light yellow powder, soluble in water, methanol, ethanol and butanol containing water, but insoluble in benzene, ether, petroleum ether, chloroform and acetone. Its absorption spectrum can be characterized as by showing no other maximum except that at 216 mμ. Fraction B is a colorless liquid (b.p. 36°C at 1/1000 mg Hg) and soluble in various organic solvents such as methanol, ethanol, benzene, ether, acetone, petroleum ether and chloroform, but insoluble in water. Fraction C is a light reddish-brown powder, soluble in ether, acetone and chloroform, but insoluble in water, benzene and petroleum ether.

The antifungal spectra of blasticidin, A, B and C against various fungi and phytopathogenic fungi by the agar streak dilution method are shown in Table V.

Summary

From taxonomic study of the strain 2A–327 which produces antibiotics showing an especially inhibitory activity against *Piricularia oryzae in vitro* and *in vivo*, it was seemed that no species described in Bergey's Manual could agree with this. So this strain was named *Streptomyces griseochromogenes* Fukunaga. Czapek's medium proved to be most suitable as for active substance production by shaking culture.

The three fraction all showing growth inhibitory activity against various fungi, yeast and phytopathogenic fungi were obtained from the culture filtrate. They are seemed to differ from the known antibiotics shown in previous literature, in regard to ultraviolet absorption, solubility, paper chromatogram and antimicrobial spectrum. Therefore, they are named blasticidin A, B and C.

The authors wish to express their cordial hearty thanks to Prof. Y. Sumiki, Prof. K. Sakaguchi, Prof. T. Asai, Assist. Prof. H. Yonehara, and H. Sakai for their guidance throughout this work.

Isolation and Identification of α-Spinasterol and α-Spinasteryl D-Glucoside from Sugar Beet Pulp.

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Received July 16, 1955

A sterol and a steryl glucoside were isolated from dried beet pulp. The sterol was identified with α-spinasterol, the glucoside possessed chemical and physical properties such as follows: The molecular formula $C_{35}H_{58}O_6$, m.p. 292°, $[\alpha]_D^{19} -34.1°$, acetate; m.p. 168°, benzoate; m.p. 175–177°, and positive for Molish and Liebermann-Burchard reactions. When it was hydrolyzed with 1% sulfuric acid, the crystal of α-spinasterol and D-glucose detectable by paper chromatography were obtained. These results gave evidence that the glucoside was in question to be α-spinasteryl D-glucoside.

Introduction

There have been two investigations on the sterol of beet. Namely, both Neville[1] and Pavlas[2] obtained the crystals of sterol from beet respectively, their crystals were characterized as the sterol by color reactions. The present authors attempted to isolate and identify the sterol from beet pulp (*Beta vulgaris*), and found them to be identical with α-spinasterol[3], which was first isolated from spinach[3], and α-spinasteryl D-glucoside which never seemed to have been found in a state of nature.

Experimental

Isolation of α-Spinasterol and α-Spinasteryl D-Glucoside.

About 10 kg. of dried beet pulp was extracted with boiling alcohol, and the extract was concentrated under a reduced pressure. This extract was dissolved in a small quantity of boiling 95% alcohol, and the crude α-spinasteryl D-glucoside which separated, was filtered and washed with ether. After recrystallization three times from pyridine, the glucoside was obtained in the form of plates melting at 292°. The yield was about 2.0 g. It gave positive Liebermann-Burchard and Molish reactions. $[\alpha]_D^{19} -34.1°$ (pyridine, 1.186 g/100 ml. l=2 dm.).

Anal. Found: C, 73.09; H, 10.04, Calcd. for $C_{35}H_{58}O_6$: C, 73.13; H, 10.10.

The alcohol and ether were then removed by distillation. The residue was saponified with 5% alcoholic-KOH solution, and the unsaponifiable fraction was continuously extracted with ether. α-Spinasterol was recovered when the ether was removed by distillation. The sterol was filtered and recrystallized from acetone, ethyl acetate and ethanol; m.p. 167–168°, yield about 4.0 g. The melting point of a mixture with α-spinasterol[4] was 167–168°. $[\alpha]_D^{19} -2.5°$ (chloroform, 2.590 g/100 ml. l=2dm.)

Anal. Found: C, 84.07; H, 11.50, Calcd. for $C_{29}H_{48}O$: C, 84.38; H, 11.73.

α-Spinasteryl Acetate

A sample of 138 mg. of the α-spinasterol described above was dissolved in 5 ml. of pyridine and 10 ml. of acetic anhydride was added. The mixture was refluxed for an hour and then poured into water. The precipitate was filtered and recrystallized twice from 95% ethanol; yield 150 mg.. m.p. 179–181°, mixed with authentic sterol (183–185°) 181–182°.

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[1] Allen Neville, *J. Chem. Soc.* (*London*), 101, 1101 (1912).

[2] Petre Pavlas, *Listy Cukrovar*, **47**, 527 (1929).

[3] F.W. Heyl, E.C. Wise and J.H. Speer, *J. Biol, Chem.*, **82**, 111 (1929). **95**, 311 (1932).

[4] K. Takeda, *J. Pharm. Soc. Japan*, **73**, 272 (1953).

Anal. Found: C, 81.52; H, 11.08, Calcd. for $C_{31}H_{50}O_2$: C, 81.88; H, 11.08.

Saponification of the acetate regenerated the α-spinasterol (m.p. 167–168°).

α-Spinasteryl Benzoate.

A sample of 250 mg. of the α-spinasterol was dissolved in 2 ml. of pyridine, and 0.5 ml. of benzoyl chloride was added. The mixture was heated for two hours and allowed to stand overnight. The benzoate was recrystallized thrice from 95% ethanol; yield 125 mg., m.p. 198–200°, mixed with authentic benzoate (198–200°), also 198–200°.

Anal. Found: C, 83.47; H, 10.01, Calcd. for $C_{36}H_{52}O_2$: C, 83.65; H, 10.15.

α-Spinasteryl D-Glucoside Acetate.

A sample of 38 mg. of the α-spinasteryl D-glucoside described above was dissolved in 5 ml. of pyridine and 5 ml. of acetic anhydride was added. The mixture was refluxed for an hour and poured into water. The precipitate was filtered and recrystallized twice from ethanol; yield 42 mg., m.p. 168°.

α-Spinasteryl D-Glucoside Benzoate.

A sample of 100 mg. of the glucoside was dissolved in 1 ml. of pyridine and 0.5 ml. of benzoyl chloride was added. The mixture was refluxed for two hours and poured into water. The precipitate was filtered and recrystallized thrice from 95% ethanol; yield 78 mg., m.p. 175–177°.

Hydrolysis of α-Spinasteryl D-Glucoside.

A sample of 533 mg. of the purified α-spinasteryl D-glucoside was hydrolyzed by the method of Thornton, et al.[5]

The glucoside was refluxed for twenty-four hours with 100 ml. of absolute ethanol and 1ml. of concentrated sulfuric acid, the solution gradually taking as the reaction progressed. The bulk of the ethanol was distilled off under a reduced pressure and the residue was diluted with water and extracted with several portions of ether.

The ethereal solution was washed with water and washings added to the main solution which was then further acidified with 1ml. of concentrated sulfuric acid and refluxed for five hours to hydrolyze ethyl glucoside. After the sulfuric acid was removed with barium carbonate, the aqueous solution was concentrated under a reduced pressure. This solution was used to establish the identity of the sugar.

α-Spinasterol Component of the Glucoside.

The washed ether extract of the hydrolysed mixture was evaporated. The recovered sterol was recrystallized from the ethyl acetate and 95% ethanol; m.p. 166.5–168°; mixed with α-spinasterol was 166–168°.

A portion of the sterol was acetylated with pyridine and acetic anhydride in the usual way. The acetate was recrystallized thrice from 95% ethanol; m.p. 177–178°.

Anal. Found: C, 81.88; H, 10.91, Calcd. for $C_{31}H_{50}O_2$: C, 81.88; H, 11.08.

Identification of D-Glucose from the Glucoside.

The aqueous solution reduced the Fehling solution and the Tollens reagent. Glucose was identified by paper chromatography, developing with phenol saturated with water and *n*-butanol-acetic acid-water, (4:1:2) and spraying with aniline phthalate and ammoniacal silver nitrate. It was compared with the authentic L-arabinose, D-xylose, L-rhamnose, D-fructose, D-mannose, D-galactose and D-glucose, and was found to be identical with D-glucose.

Infrared Data

The α-spinasterol was compared with the data of Rosenkrantz.[6]

In the range of 2–15μ, the infrared curves were found qualitatively identical in every detail. Sample bands occured at 3.00 (bonded hydroxyl), 3.43, 3.50, 6.02, 6.11, 6.84, 7.24, 8.00, 8.19, 8.36, 8.69, 8.92, 9.09, 9.34, 9.56, 9.62, 9.85, 10.69, 11.23, 11.49, 11.90, 12.04, 12.65, 12.98μ.

The bands of α-spinasteryl D-glucoside occured at 6.84, 7.24, 7.51, 7.63, 7.81, 7.96, 8.19, 8.29

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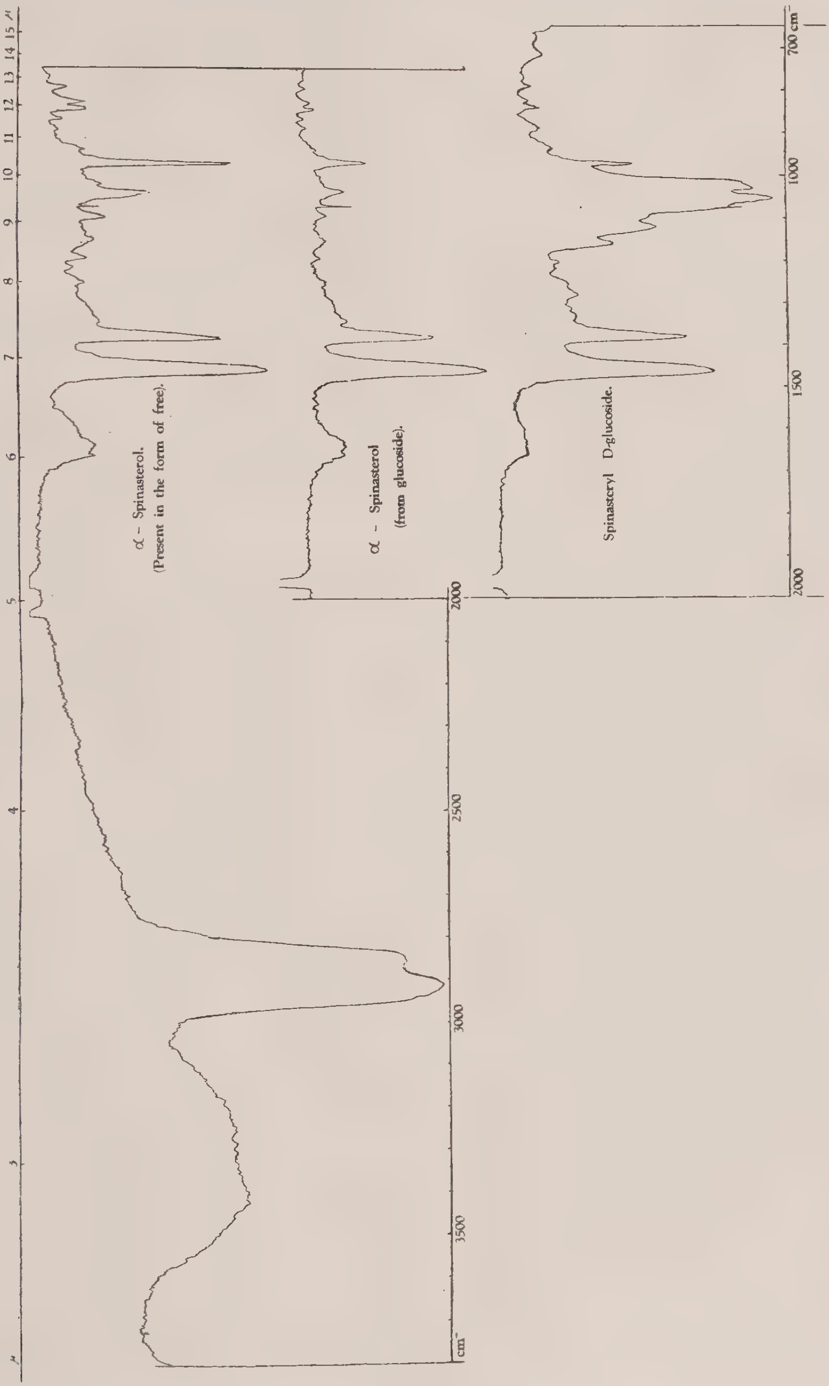
[5] M. H. Thornton, H. R. Kraybill and J.H. Mitchel, Jr. *J. Am. Chem. Soc.* **62**, 2006 (1940).

[6] H. Rosenkrantz, et al,, *J. Biol, Chem.*, **195**, 503 (1952).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1000
700 cm⁻¹
α - Spinasterol.
(Present in the form of free).
α - Spinasterol
(from glucoside).
Spinasteryl D-glucoside.

8.54, 8.92, 9.30, 9.47, 9.66, 10.30, 10.69, 11.23, 11.90, 12,12, 12.65, 12.90, 13.98μ.

Summary

α-Spinasterol and α-spinasteryl D-glucoside were isolated from sugar beet pulp. The α-spinasteryl D-glucoside was hydrolyzed, and each component was identified with α-spinasterol and D-glucose respectively. Infrared data showed that α-spinasterol was identical with that isolated from spinach. α-Spinasteryl D-glucoside has been found for the first time in natue.

We are indebted to Mr. K. Takeda for kindly supplying the sample of α-spinasterol, to the Laboratory of Microanalyse, University of Tokyo for the analytical results and also to the Central Research Institute of the Japan Monopoly Corporation for infrared data.

Studies on Synthetic Pyrethroids. Part V. Synthesis of Geometrical Isomers of Chrysanthemum Dicarboxylic Acid.[*]

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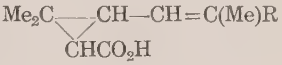
Received July 19, 1955

Addition of ethyl diazoacetate to ethyl α,δ-dimethyl-*trans*-sorbate gives adducts, and hydrolyses of these esters yield (±)-*trans*-, (±)-*cis*-3-(*trans*-2′-carboxypropenyl)-2,2-dimethyl-cyclopropane-1-carboxylic acids and α,δ-dimethyl-γ-carboxymethyl-*trans*-sorbic acid. Their structures and configurations are confirmed and (±)-*trans*-3-(*trans*-2′-carboxypropenyl)-2,2-dimethylcyclopropane-1-carboxylic acid is shown to be the racemic form of the natural chrysanthemum dicarboxylic acid. Thus the geometrical configuration of the side chain of the chrysanthemum dicarboxylic acid is considered to be *trans*.

Chrysanthemum dicarboxylic acid was at first isolated from Japanese pyrethrum flowers by J. Fujitani[4] as early as in 1909. This crystalline dextrorotatory dicarboxylic acid was also obtained by H. Staudinger and L. Ruzicka[5] together with the monomethyl-(i.e. pyrethric acid) and dimethylesters, from the cold sodium methylate fission of the mixed semicarbazones of the 'pyrethrins', and was found to be an acidic component of pyrethrin II and cinerin II. The structure of the dicarboxylic acid was established as (**I**, R = CO_2H) from ozonisation which gave (−)-*trans*-caronic acid and pyruvic acid, while that of pyrethric acid as (**I**, R = CO_2Me), similarly from the isolation of (−)-*trans*-caronic acid and methyl pyruvate.



$$\underset{\mathrm{CHCO_2H}}{\mathrm{Me_2C{-}CH}}\mathrm{{-}CH{=}C(Me)R}$$

(I)

It was shown that this dicarboxylic acid had the same *trans* configuration as chrysanthemic acid about the cyclopropane ring, but the configuration of the side chain remained unknown and would be confirmed by synthesis or physical methods, because the double bond in the side chain resists hydrogenation, and bromine is added very slowly.

The similarity in structure between chrysanthemum mono- and dicarboxylic acids suggested that the method developed by Harper[6] with success for the synthesis of the former, might serve a possible route to the latter and it appeared of interest to undertake its synthesis.

In the first attempt,[1] ethyl α,δ-dimethyl-β-hydroxy-γ-hexenoate (**III**), prepared by the Reformatski condensation of β-methylcrotonaldehyde (**II**) and ethyl α-bromopropionate, was used as an olefinic component for the addition of ethyl diazoacetate.

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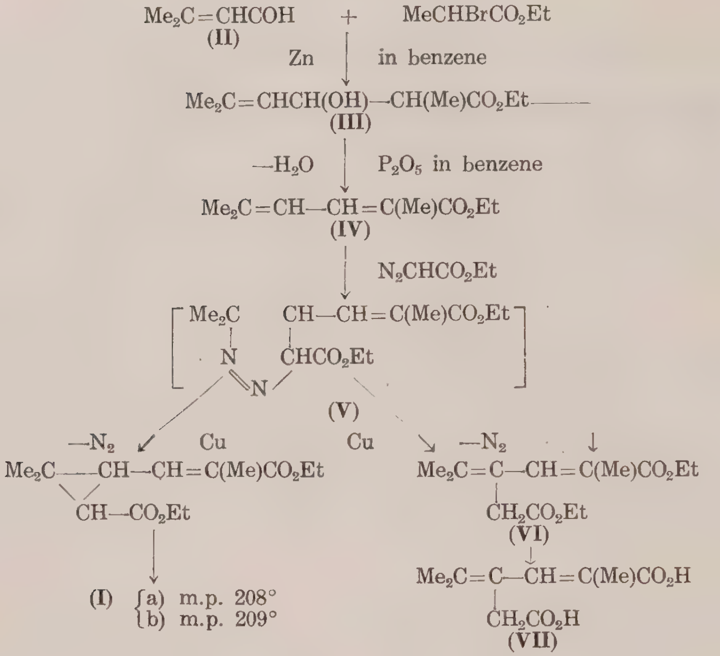
[*] Part IV. *Botyu Kagaku* **19**, 102 (1954). Short notes on this work appeared in (1) *Botyu Kagaku* **19**, 35 (1954), (2) *Bl. Inst. Chem. Res. Kyoto Univ.* **33**, 73 (1955), (3) Report to the Ministry of Education, Oct. 1954, and was presented at 112th Meeting of the Agr. Chem. Soc. Japan, Kansai Branch, July 1954 and at the annual meeting of the Agr. Chem. Soc. Japan, March 1955.

[4] Fujitani, *Arch. exp. Path. Pharm.* **61**, 47 (1909).

[5] Staudinger, Ruzicka, *Helv.* **7**, 201 (1924).

[6] Harper, *J. Chem. Soc.* **1945**, 283.



At first sight it seemed likely that ethyl diazoacetate could only add at the γ,δ-ethylenic bond in the Reformatski ester (**III**), giving cyclopropane derivative, followed by dehydration to produce the synthetic chrysanthemum dicarboxylic acid desired. Investigation showed that the addition reaction occurred at the γ,δ-position as expected, but the possible intermediate pyrazoline decomposed in a manner to give an acyclic isomeric acid (**VII**), which will be discussed later. Attempts to obtain cyclopropane compounds through this route by employing suitable condition ended in failure and it might contribute little to the elucidation of the stereochemistry of the side chain.

Thus, our attention was directed to ethyl α,δ-dimethylsorbate (**IV**), obtained from the dehydration of the Reformatski ester (**III**), as the olefinic component required, because of the resemblance to dimethylhexadiene in the case of chrysanthemic acid. In the condensation of diazoacetate and dimethylsorbate (route B), however, the sorbate contains two ethylenic bonds in molecule and lacks symmetry, and successful synthesis of (**I**) would postulate the addition of diazoacetate at the γ,δ-ethylenic bond. Available experimental data concerning the addition of aliphatic diazoacetate to asymmetrical diene systems, substituted by polar groups, are not sufficient to make a confident prediction on this point.⁷⁾

It has been well known that ethylenic bond, when substituted by negative groups, behaves less unsaturated as compared with simple olefinic bond, and that the δ-lactonization is apt to take place in alkyl substituted sorbic acids.⁸⁾ Therefore, it looked probable that addition of ethyl diazoacetate would take place at

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(7) Guha, *Ber.* **70**, 2109 (1937).

(8) Reformatski, *Ber.* **28**, 2842 (1895), Fichter, *ibid.* **42**, 4710 (1909), Willstaetter, *Ann.* **418**, 148 (1919).

γ,δ-rather than at α,β-position in dimethylsorbate.

Preliminary experiments⁹⁾ on the addition of diazoacetate to methyl or ethyl α,β,δ-trimethylsorbate, prepared from the Reformatski condensation of readily available mesityloxide with α-bromopropionate, showed the formation of cyclopropane acids resulted from γ,δ-addition. Furthermore, the fact that the acyclic acid (**VII**) which was at first obtained by the condensation of diazoacetate with the Reformatski ester (**III**) (route A), was also prepared by the addition to dimethylsorbate (**IV**), suggested the possibility that this reaction would procede in a way to give cyclopropane derivative if other suitable condition were employed.

The Reformatski condensation of β-methylcrotonaldehyde (**II**), prepared by the method described in the preceding paper (Part IV), with ethyl α-bromopropionate gave the β-hydroxy ester (**III**) and this was dehydrated with phosphorus pentoxide or phosphoric oxychloride in dry benzene to yield an unsaturated ester. Hydrolysis of this ester with alcoholic potassium hydroxide gave the corresponding acid, $C_8H_{12}O_2$, which melted at 133–4°. Both the ester and acid were readily hydrogenated over platinum oxide catalyst, two mols. of hydrogen being taken up, to give ethyl α,δ-dimethylcaproate and the corresponding acid. This was characterised by amide and *p*-phenylphenacyl ester. Ultra-violet absorption (λ_{max} 273 mμ, ε 22,300) as well as the catalytic hydrogenation, showed the unsaturated acid to be the required α,δ-dimethylsorbic acid. This was the only product obtained by dehydration of the Reformatski ester. When hydroxy compounds, such as derived from the Reformatski and the Grignard reactions, are dehydrated with chemical reagents at a comparatively lower temperature, the mixture of geometrical isomers is to be expected, even if the dehydration is stereospecific. Sometimes, however, only a *trans* isomer has been isolated,¹⁰⁾ as is the case with ours. This may be due to stereomutation or to experimental difficulties. There is steric hindrance in the model of *cis*-form and would be expected to be less stable, and both the known form and the partialy reduced α,β-unsaturated acid are characteristic of acids of *trans* configuration.

Therefore, the configuration of the only known form of α,δ-dimethyl sorbic acid is considered to be *trans*. The absence of *cis*-form may be due to stereomutation during the process of dehydration. Evidences in support of this view are to be advanced in a succeeding paper.

When ethyl diazoacetate was added to equimolar ethyl α,δ-dimethylsorbate in the presence of copper powder under a violent condition, an adduct ester was produced, which, on hydrolysis, gave the diacid $C_{10}H_{14}O_4$, m.p. 185–6°, idential with that prepared from the Reformatski ester (**III**) (route A).¹⁾ This shows the addition at γ,δ-position, but the infra-red spectra having no absorption in the region 9.8–10μ in which alkyl substituted cyclopropane derivatives show a strong band.¹¹⁾ Absorption of two moles of hydrogen upon catalytic reduction and the ultraviolet absorption (λ_{max} 274 mμ, ε 29,700) indicate the same alkyl substituted conjugated diene carboxylic system in this molecule as in the original sorbic acid.

This excludes the combination of diazo-

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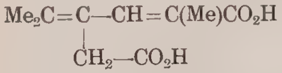
(9) unpublished.

(10) Lutz, *J. Am, Chem. Soc.* **74**, 479 (1949), Grummitt, *ibid.* **73**, 3479 (1951) Fieser, *Experimentia*, **4**, 285 (1948).

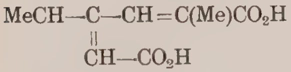
(11) Defer, *J. Am. Chem. Soc.* **71**, 2482, 3595 (1949).

acetate residue to δ-carbon atom and thus, the structures (**VIII**) and (**IX**) are possible for this acyclic dicarboxylic acid.



$$\mathrm{Me_2C{=}\underset{\displaystyle{|}\atop\displaystyle CH_2{-}CO_2H}{C}{-}CH{=}C(Me)CO_2H}$$

(VIII)



$$\mathrm{MeCH{-}\underset{\displaystyle{\|}\atop\displaystyle CH{-}CO_2H}{C}{-}CH{=}C(Me)CO_2H}$$

(IX)

Of the two, only (**VIII**) can give rise to acetone on ozonisation, which was isolated as a 2,4-dinitrophenylhydrazone, together with pyruvic acid and an acid suggesting oxalacetic acid.

The formation of (**VII**) by the route A, and the steric hindrance in the model of *cis*-form suggest the retention of the *trans* configuration of carboxypropenyl group throughout the reaction.

This seems to be the case also in the addition of ethyl diazoacetate to ethyl α,δ-dimethylsorbate in a way to yield cyclopropane derivatives, which are discussed below.

From the adduct, resulted by the addition of ethyl diazoacetate to (**IV**) in large excess or diluted with ligroin under milder conditions, the crystalline acids, m.ps. 207–8°* and 208–9°, were isolated by repeated separate crystallizations from dilute alcohol.

Ultra-violet absorption of the former acid (Ia) ($\lambda_{\max}$ 237 mμ, ε 15,400) indicated the same conjugated cyclopropane carboxylic acid system in the molecule as the natural chrysanthemum dicarboxylic acid ($\lambda_{\max}$ 238 mμ, ε 15,300). Upon ozonisation (Ia) gave (±)-*trans* caronic acid, m.p. 212°, and pyruvic acid (as a 2,4-dinitrophenylhydrazone m.p. 218°) and the comparison with authentic specimens resulted no depression of m.ps. Infra-red spetra in Nujol mull (Fig. 1) was perfectly identical with that of natural chrysanthemum dicarboxylic acid.

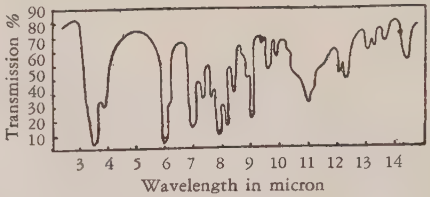


Fig. 1. Infra-red spectra of (±)-*trans*-3-(*trans*-2′-carboxypropenyl)-2, 2-dimethylcyclopropane-1-carboxylic acid (in line) and natural chrysanthemum dicarboxylic acid (in dotted line in Nujol mull.

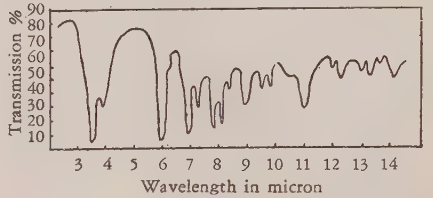


Fig. 2. Infra-red spectra of (±)-*cis*-3-(*trans*-2′-carboxypropenyl)-2,2-dimethylcyclopropane -1-carboxylic acid in Nujol mull.

Thus, this (±)-*trans*-3-(*trans*-2′-carboxypropenyl)-2,2-dimethylcyclopropane-1-carboxylic acid is the racemic form of the natural chrysanthemum dicarboxylic acid.

The latter acid (Ib) showed a depression of m.p. when mixed with the former (Ia), and gave (±)-*cis* caronic acid m.p. 174°, as well as pyruvic acid as a 2,4-dinitrophenylhydrazone upon ozonisation. Infra-red spectra of (Ib) in Nujol mull (Fig. 2) was very similar to those of (Ia) and of natural acid, but ultra-violet absorption ($\lambda_{\max}$ 242 mμ, ε 15,700) was not identical. Thus, this (±)-*cis*-3-(*trans*-2′-carboxypropenyl)-2, 2-dimethylcyclopropane-1-carboxylic acid is the *cis* isomer of (Ia) with reference to cyclopropane ring.

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* We learnt by Prof. S. H. Harper's communication, that he also had synthesised the geometrical isomers of chrysanthemum dicarboxylic acid by the same routes. (Chem. & Ind. **1954**, 1538 9).

Concerning the mechanism of the addition of diazoacetate to sorbate, the assumption of the possible intermediate pyrazoline (**V**) would be more reasonable than the free radical mechanism of ethoxycarbonylmethylene radical for the elucidation of the formation of acyclic compound (**VII**) and cyclopropane derivatives (**Ia**, **Ib**), as well as the formation of cyclopropane derivatives resulted from inversion of γ,δ-olefinic bond of ethyl sorbate and α-methylsorbate.[12]

Experimental

All melting points and boiling points were uncorrected. Ultra-violet absorptions were determined in ethanol on a Beckmann model DU quartz spectrophotometer. Infra-red spectra were recorded in Nujol mull on a Perkin Elmer double beam recording spectrophotometer. Microanalyses were by Microanalytical Division, Prof. Mitsui Laboratory.

(1) Ethyl α,δ-Dimethyl-β-hydroxy-Δγ-hexenoate (III)

About 50 ml. of a mixture of β-methylcrotonaldehyde (42 g. 0.5 mol), prepared by the method described in Part IV, ethyl α-bromopropionate (91 g. 0.5 mol) and 90 ml. of dry benzene was added on 33 g. (0.5 atom) of activated zinc and it was warmed with effective stirring until reaction set in. Addition of a trace of mercuric chloride initiates the reaction smoothly. The remaining mixture was then run in at such a rate that the refluxing benzene was under control. It was then warmed under reflux for an additional half an hour on a water bath, cooled and poured into an ice cold solution of dilute sulfuric acid. After removal of the unchanged zinc, the organic layer was washed with small portions of water and dried over anhydrous magnesium sulfate. The benzene was removed through a column, and the residue fractionated under a reduced pressure to give a fraction b.p. 84–5°/4 mm, 52 g. n_D^{15} 1.4580. This Reformatski ester gave on hydrolysis a viscous liquid acid. *p*-Phenylphenacylester m.p. 82–3°, *Anal*. Found: C, 74.53; H, 6.84; Calcd. for $C_{22}H_{24}O_4$: C, 74.97; H, 6.86%. *p*-Iodophenacylester m.p. 107–8°, *Anal*. Found: C, 47.70; H, 4.72; Calcd. for $C_{16}H_{19}O_4I$: C, 47.77; H, 4.76%.

(2) Ethyl α,δ-Dimethyl-*trans*-sorbate (IV)

Fifty six grams (0.3 mol) of the Reformatski ester (**III**) was diluted with three volumes of dry benzene and boiled under reflux for 1.5 hours with slight excess of phosphorus pentoxide (45 g) or phosphoric oxychloride. Water was then added, and the ester isolated in usual way, b.p. 85–6°/6 mm. 32 g. n_D^{20} 1.5015. Hydrolysis of the ester with 10% alcoholic potassium hydroxide gave an acid, m.p. 133–4°, $\lambda_{\max}$ 273 mμ, ε 22,300, *Anal*. Found: C, 68.44; H, 8.69; Calcd. for $C_8H_{12}O_2$: C, 68.54; H. 8.63%. *p*-Phenylphenacylester m.p. 102–3°, *Anal*. Found: C, 79.13; H, 6.64; Calcd. for $C_{22}H_{22}O_3$: C, 79.01, H, 6.63%. Upon hydrogenation over platinum oxide catalyst this acid absorbed 1.97 mol. hydrogen, yielding a saturated acid, α,δ-dimethylcaproic acid, b.p. 115–6°/13 mm., n_D^{20} 1.4261. Amide, m.p. 102–3°, *Anal*. Found: C, 67.18; H, 12.01; Calcd. for $C_8H_{17}ON$: C, 67.09; H, 11.96%. *p*-Phenylphenacylester m.p. 66°, *Anal*. Found: C, 78.31; H. 7.91; Calcd. for $C_{22}H_{26}O_3$: C, 78.09; H. 7.74%.

(3) α,δ-Dimethyl-γ-carboxymethyl-*trans*-sorbic acid (VII)

a) Addition of ethyl diazoacetate to the Reformatski ester (**III**) was performed in the presence of copper powder at 130–140° in a nitrogen atmosphere. The resulting adduct was treated with phosphorus pentoxide in dry benzene and hydrolysed with alcoholic potassium hydroxide. An acid melting at 185–6° was obtained in a poor yield.

b) Addition of ethyl diazoacetate to ethyl α,δ-dimethyl-*trans*-sorbate. A mixture of ethyl diazoacetate (14 g. 0.12 mol) and ethyl α,δ-dimethyl-*trans*-sorbate (20 g. 0.12 mol) was slowly added to a suspension of copper powder (0.5 g) in a small amount of sorbate, preheated to about 110°. A violent reaction occurred with a vigorous evolution of nitrogen and the rate of addition was adjusted so that the reaction temperature was maintained at 110–130°. The distillation of the adduct gave a fraction, b.p. 91–2°/0.05 mm. 9.5 g. n_D^{15} 1.4725, and hydrolysis of the ester

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(12) Harper, *J. Chem. Soc.* **1955**, 779.

with alcoholic potassium hydroxide yielded the acid, m.p. 185–6°, (VII), 3 g. λ_{max} 274 mμ, ε 29,700 *Anal.* Found: C, 60.69; H, 7.05; Calcd. for $C_{10}H_{14}O_4$: C, 60.59; H, 7.12%. *p*-Phenylphenacylester, m.p. 150–51°, *Anal.* Found: C, 77.94; H. 5.86; Calcd. for $C_{38}H_{34}O_6$: C, 77.79, H. 5.84%. The infrared spectra showed the absence of characteristic band of cyclopropane.

Ozonisation—The acid (0.5 g), m.p. 185–6°, in chloroform was treated with excess of ozone at 0°. The solvent was removed in a vacuum, and the remaining ozonide was decomposed with water on a water bath for ten minutes. The solution was distilled at first in an atmospheric pressure and then under reduced pressure. From the first distillate, acetone was isolated as a 2,4-dinitrophenylhydrazone, m.p. 127–8°, (*Anal.* Found: C, 45.67; H, 4.24; Calcd. for $C_9H_{10}O_4N_4$: C, 45.38; H, 4.23%) and from the second distillate, pyruvic acid as a 2,4-dinitrophenylhydrazone, m.p. 216°, (*Anal.* Found: C, 40.36; H, 3.06; Calcd. for $C_9H_8O_6N_4$: C, 40.30, H, 3.01%), not depressed by each authentic specimen of the same m.p. The residue, when derived to phenacylester, gave a *p*-phenylphenacylester, m.p. 119–20°. *Anal.* Found: C, 72.80; H, 5.11; Calcd. for di-*p*-phenylphenacylester of oxalacetic acid, $C_{32}H_{24}O_7$: C, 73.84; H, 4.65%.

Hydrogenation—The acid (0.4 g) in 10 ml. alcohol was hydrogenated over Adams platinum oxide catalyst in a shaking hydrogenation apparatus, and absorbed 95 ml. (at 20°) of hydrogen, giving a saturated acid, α-methyl-γ-isopropyladipic acid, m.p. 94–5°. *Anal.* Found: C, 59.16; H, 8.68; Calcd. for $C_{10}H_{18}O_4$: C, 59.38; H, 8.97%. Di-*p*-phenylphenacylester, m.p. 106–7°, *Anal.* Found: C, 77.39; H, 6.22; Calcd. for $C_{38}H_{83}O$: C, 77.28; H, 6.48%.

(4) Synthetic Chrysanthemum Dicarboxylic acids (Ia, Ib)

a) Ethyl diazoacetate (10 g) was added slowly to a large excess of ethyl α,δ-dimethyl-*trans*-sorbate (60 g) at 80–90° and this temperature was maintained for several hours, and then the reaction mixture was gently heated to 120° with two grams of copper powder until evolution of nitrogen ceased. The unchanged sorbate was recovered by distillation under a reduced pressure and the residue was hydrolysed with alcoholic potassium hydroxide, giving two crystalline acids of m.ps. 207–8° and 208–9° (see below).

b) About 1 ml. of ethyl diazoacetate was added to a solution of ethyl α,δ-dimethyl-*trans*-sorbate (42 g) in equal volume of ligroin containing 1 g. of copper powder, and the mixture heated until reaction set in with the evolution of nitrogen (temperature 125–130°). The remaining diazoacetate (15 g) was then added at such a rate that the steady reflux and the smooth evolution of nitrogen maintained, without external application of heat. After the removal of the solvent and unchanged sorbate by distillation under a reduced pressure, the oily residue was hydrolysed with alcoholic potassium hydroxide, giving the adduct acids in oil. This was extracted with dilute ethanol and a crystalline mass separated out slowly from the concentrate of the extract. Repeated separate crystallisations from dilute ethanol yielded the first product melting at 207–8°, (±)-*trans*-3-(*trans*-2′-carboxypropenyl)-2,2-dimethylcyclopropane-1-carboxylic acid. *Anal.* Found: C, 60.63; H, 7.11; Calcd. for $C_{10}H_{14}O_4$: C, 60.59, H, 7.12%, equiv. found 99.5, calcd. 99.1 λ_{max} 237 mμ, ε 15,400. Natural chrysanthemum dicarboxylic acid λ_{max} 238 mμ, ε 15,300. Infrared spectra see Fig. 1 in text.

Ozonisation—The acid (0.8 g) in chloroform (40 ml) was treated with excess of ozone for several hours at 0°. The solvent was removed at 25° under a reduced pressure, and the oily ozonide was decomposed with addition of water by warming on a water bath for ten minutes. The resulting aqueous solution was then distilled under a reduced pressure. From the distillate caught in a chilled receiver, pyruvic acid was isolated as a 2,4-dinitrophenylhydrazone, m.p. 218°, *Anal.* Found: C, 40.39; H, 3.10; Calcd. for $C_9H_8O_6N_4$: C, 40.30; H, 3.01%. (±)-*trans* Caronic acid (310 mg) m.p. 212°, separated out from the residue. *Anal.* Found: C, 53.36; H. 6.21; Calcd. for $C_7H_{10}O_4$: C, 53,16; H, 6.37%. Mixed melting point comparison of these compounds with the respective authentic specimen resulted no depression.

The second acid was obtained from the filtrate of the crystallisation of the *trans* acid and recrystallisation from dilute methanol yielded (±)-*cis*-3-(*trans*-2′-carboxypropenyl)-2,2-dimethylcyclopropane-1-carboxylic acid, m.p. 208–9°, which resulted remarkable depression of m.p. when mixed with the former acid. *Anal.* Found: C, 60.60; H, 7.07; Calcd. for $C_{10}H_{14}O_4$: C, 60.59; H, 7.12%. equiv. found 99.7, calcd. 99.1, λ_{max} 242 mμ, ε 15,700 Infra-red spectra see Fig. 2 in text.

Ozonisation procedure of this acid (0.6 g) was exactly the same as described above and (±)-*cis*-caronic acid, m.p. 173–4°, (200 mg), (*Anal.* Found: C, 53.40; H, 6.44, Calcd. for $C_7H_{10}O_4$ C,: 53.16; H, 6.37%) and pyruvic acid as 2,4-dinitrophenylhydrazone, m.p. 218°, were obtained. *Anal.* Found: C, 40.21; H, 3.08; Calcd. for $C_9H_8O_6N_4$: C, 40.30; H, 3.01%.

These resulted no depression of melting points when mixed with authentic specimens.

The authors are indebted to Prof. Dr. S. Takei for his kind advices and suggestions. This work was supported in part by a research grant from the Ministry of Education.

Crystallization of Animal Catalase and Studies on its Optimum Temperature.

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Received August 11, 1955

Optimum temperatures of the animal catalases extracted from several different sources are not identical.

The problem of what factors cause this difference, has recently been studied on other enzymes by a few investigators, and they have reported that various factors exert effect on the optimum temperatures of enzymes.

The enzyme preparations used by them were, however, not so pure such as the crystalline ones, so that the results obtained had many doubtful points, consequently, the influence of accompanied ions or protective proteins was presumed.

Now, we prepared the crystalline catalases from both cow and toad livers, and after measuring the optimum temperature at each purification stage, we found out that the optimum temperature was independent of enzyme purity.

From the above results, it may be deduced that the nature of each protein part of catalase isolated from various sources are not identical and this makes the optimum temperatures of enzymes specific to each organism.

Since Sumner and Dounce[1] succeeded in crystallizing cow liver catalase in 1937, animal catalases have been isolated from several different sources in crystalline forms.

For example, this enzyme has been crystallized from horse liver[2], sheep liver[3], rat liver[4] and beef erythrocytes[5]. Moreover, it was also purified from a bacterium[6]. It is, nowadays, believed that the animal catalase can be most easily crystallized.

It has then become a very interesting problem to investigate whether each crystalline catalase have the identical property or not, as we could prepare highly purified enzymes from various sources.

From this view-point, Herbert and Pinsent[6] have isolated bacterial catalase in a crystalline form.

Having the same purpose, we have been making attempts to crystallize catalases from animal, yeast and plant.

In the following paper, we succeeded in crystallizing plant catalase which had been deemed very difficult to crystallize and in purifying yeast catalase to a certain degree.

Furthermore, it is conceivable that even among the animal catalases, there may be difference in their properties such as those observed among animal, yeast and plant catalases.

It is obvious that animal catalase has haemin as its prosthetic group, but the protein parts of each catalase may be different, according to their sources.

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[1] J.B. Sumner and A.L. Dounce, *J. Biol. Chem.*, **121**, 417 (1937).

[2] J.B. Sumner, A.L. Dounce and V.L. Frampton, *J. Biol. Chem.*, **136**, 343 (1940).

[3] A.L. Dounce, *J. Biol. Chem.*, **143**, 497 (1942).

[4] V.E. Price and R.E. Greenfield, *J. Biol. Chem.*, **209**, 363 (1954).

[5] M. Laskowski and J.B. Sumner, *Science*, **94**, 615 (1941).

[6] D. Herbert and J. Pinsent, *Biochem. J.*, **43**, 193 (1948).

One of us⁷⁾ found each catalase extract to have a different optimum temperature, i.e. their optimum temperatures were closely resembled to the temperature of the circumustances where they had grown or of their bodies. For example, the optimum temperature of cow liver catalase (the warm blooded animal) is 40°, but that of toad liver catalase (the cold blooded animal) is 15°.

But the enzyme solution used for these measurements was too crude to decide whether the difference was due to the protein part of the enzyme itself, or to ions or other proteins accompanied.

In order to investigate the problems mentioned above, we isolated crystalline catalases from both cow and toad livers, previous to comparing animal catalase with those of yeast or plant.

In the process of catalase isolation, we have performed electrophoretic analysis, absorption spectrum analysis and measured the optimum temperatures, and have found many interesting facts especially regarding the optimum temperature.

We should like to report our results in the following, hoping to recieve comments and criticisms from the readers.

Methods

The activity of catalase preparations was measured by the Euler and Josephson's method⁸⁾. The purity of an enzyme preparation is expressed as:

$$Kat.\text{-}f. = \frac{\text{velocity constant}}{\text{g. enzyme used in the test.}}$$

In order to obtain the dry weight of the proteins of the sample used, the sample was dialyzed four times against 500 ml. of distilled water for 24 hours. It was then dried to constant weight at 105° and weighed on a semimicro balance.

Optimum temperatures were measured by the method shown in the following. To 5 beakers, each containing 5 ml. of 0.02 N H_2O_2 in 1/90 M phosphate buffer of pH 6.8 and kept at the temperature to be tested, add 1 ml. of suitably diluted catalase solution. At every 2, 4, 6 and 8 minutes, stop the enzyme reaction with 10 ml. of 2 N H_2SO_4 and cool the beakers in an ice-water bath. Make one of the beakers a blank (zero minute), adding H_2SO_4 before addition of the enzyme solution. Then determine the remaining quantities of peroxide by iodometry and calculate the velocity constant.

Absorption spectra were measured with a Beckmann Model DU spectrophotometer, using a 1-cm Silica cell.

The Tiselius apparatus was used for electrophoretic analysis. Mobilities and apparent concentration ratios of each component were calculated at descending patterns according to Longsworth and Tiselius⁹⁾.

Experimental Results

The Method for Isolation of Catalase

We chiefly used the method of Shirakawa¹⁰⁾. In the case of toad liver catalase, average body-weight of the animals was 250 g and their livers (average liver weight of one toad is 9.5 g) were used for the isolation. The essential of the purification method adopted is given in Figs. 1 and 2.

Crystalline Form

The toad liver catalase crystallized as square thin plates as shown in Fig. 3.

This crystalline form differs from that of cow liver catalase, which according to Sumner and Dounce¹⁾ may crystallize as needles, plates or prisms, according to the crystallization conditions.

We also obtain cow liver catalase as plates, which have not a square form and are shown in Fig. 4.

Electrophoretic Analysis

We began with solution B of toad liver for

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7) H. Mitsuda: Studies on Vitamin C and Phyto-catalase (1947) K. Kondo, H. Mitsuda and F. Kawai: *Bull. Inst. Chem. Res. Kyoto Univ.*, **27**, 73 (1951).

8) H. v. Euler and K. Josephson, *Ann.*, **452**, 158 (1927).

9) L.G. Longsworth, *J. Am. Chem. Soc.*, **65**, 1755 (1943). A. Tiselius and A. Kabat, *J. Expt. Med.*, **69**, 119 (1939).

10) M. Shirakawa, *J. Agr. Chem. Soc. Japan*, **23**, 361 (1950).

Toad Liver
wash, homogenize
phosphate buffer (0.01 M pH 7.0)
extraction
centrifuge

Supernatant *Kat.-f.* **350** (solution A)
0.8 volume cold acetone
centrifuge

Precipitate
distd. H_2O
centrifuge

Supernatant *Kat.-f.* **1100** (solution B)
cryst. $(NH_4)_2SO_4$ (to 0.25 sat.)
centrifuge

Supernatant (solution C)
cryst. $(NH_4)_2SO_4$ (to 0.5 sat.)
centrifuge

Precipitate
distd. H_2O
centrifuge

Supernatant *Kat.-f.* **8600** (solution D)
stand in an ice-box

Crystalline Catalase
centrifuge

Precipitate
distd. H_2O
centrifuge

Supernatant
sat. $(NH_4)_2SO_4$ solution (to 0.5 sat.)
centrifuge

Precipitate
distd. H_2O
centrifuge

Pure Catalase Solution
Kat.-f. **21600** (solution E)

Fig. 1. Scheme for Preparing Crystalline Toad Liver Catalase.

Cow Liver
homogenize
phosphate buffer (0.01 M pH 7.0)
extraction
centrifuge

Supernatant *Kat.-f.* **530** (solution A_1)
0.8 volume cold acetone
centrifuge

Precipitate
distd. H_2O
centrifuge

Supernatant *Kat.-f.* **2300** (solution B_1)
cryst. $(NH_4)_2SO_4$ (to 0.25 sat.)
centrifuge

Supernatant
cryst. $(NH_4)_2SO_4$ (to 0.5 sat.)
centrifuge

Precipitate (solution D_1)
distd. H_2O
stand in an ice-box
centrifuge

Precipitate **(crude crystals)**
washing 3 times with H_2O
add 0.1 N NaOH until pH 7.5
centrifuge

Supernatant *Kat.-f.* **26800** (solution E_1)
make pH 5.7 with sat. KH_2PO_4 or dialyze against buffer

Crystalline Catalase
Kat.-f. **39500** (solution F_1)

Fig. 2. Scheme for Preparing Crystalline Cow Liver Catalase.

this purpose, as the extracted solution (solution A) was too turbid to analyse. And then, solution D and solution E (the most purified catalase solution) were examined. The results are shown in Fig. 5.

As it will be seen from Fig. 5, solution B has 3 peaks but the most purified solution moves as a single boundary.

Judging from the patterns and mobilities of solution E, namely, the pure catalase solution, it is clear that component 2 is a catalase fraction.

In the case of cow liver catalase, these analysis were performed at the same purification stages as those in toad liver catalase, therefore, we analysed solutions B_1, D_1, and F_1.

Fig. 6 shows the results of cow liver catalase. The experiments on cow liver catalase yielded the same results; component 2 is the catalase fraction.

Then, in Tables I and II we show the electrophoretic mobilities and apparent concentration ratios of each component of the liver catalase of both toad and cow.

The electrophoretic mobility of toad liver catalase is -2.80×10^{-5}cm^2/V sec. at pH 7.0 and that of cow liver catalase is -3.10×10^{-5}cm^2/V sec. at pH 7.0, when measured from the patterns

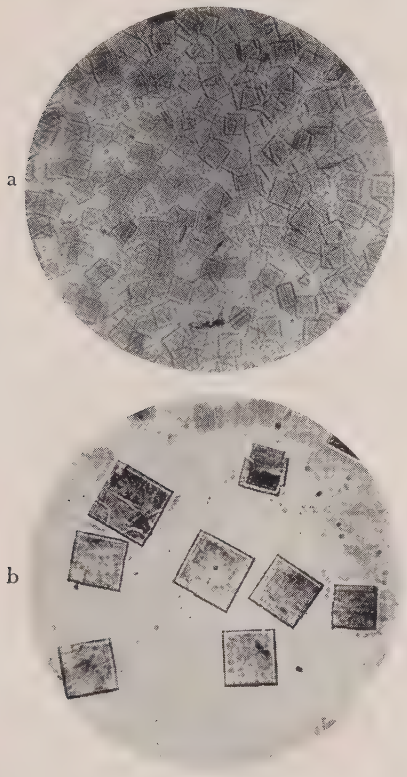

Fig. 3. Crystalline Catalase from Toad Liver.
(a ----- ×300 b ----- ×600)

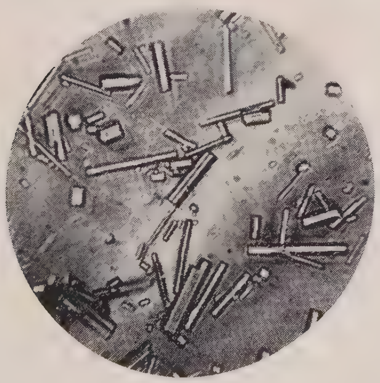

Fig. 4. Crystalline Catalase from Cow Liver.
(×400)

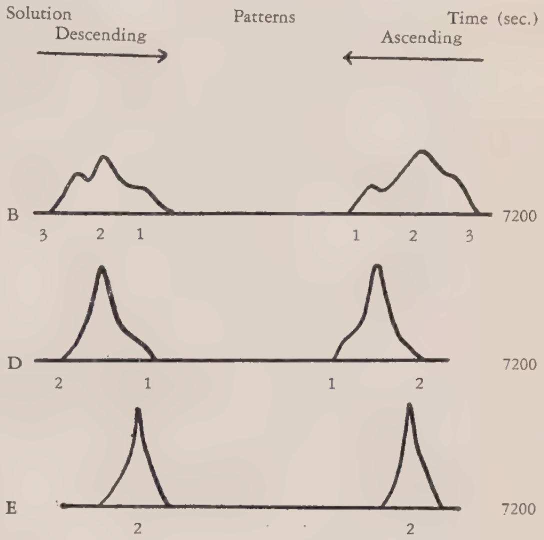

Fig. 5. Electrophoretic Patterns of Various Fractions of Toad Liver Catalases.

Electrophoresis was always performed in $\Gamma/2=0.1$, pH 7.0 N/10 phosphate buffer solution.

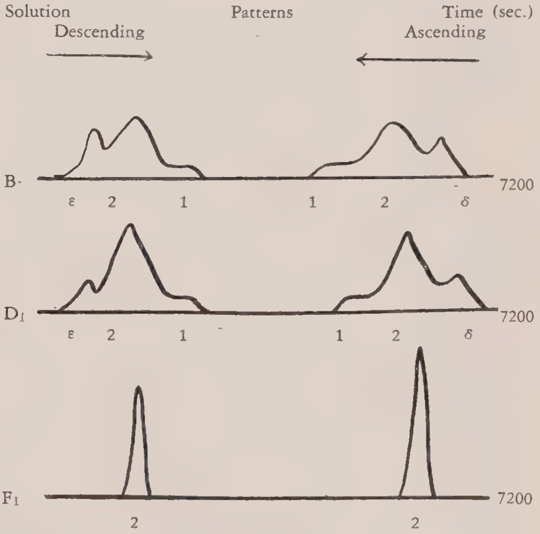

Fig. 6. Electrophoretic Patterns of Various Fractions of Cow Liver Catalases.

Electrophoresis was always performed in $\Gamma/2=0.1$, pH 7.0, N/10 phosphate buffer solution.

of pure catalase solution.

Cow liver has a large anomaly, as it may readily be seen from Fig. 6 and Table II. This anomaly seems to be such a substance like gly-

Table I

Electrophoretic Mobilities and Apparent Concentration Ratios of Component in Various Fractions of Toad Liver Catalase.

Component		1	2	3
Mobility† (mean value)		5.0	2.7	1.1
Apparent conc. ratio of comp. (%)	soln. B	21.8	53.2	25.0
〃	soln. D	14.5	85.5	—
〃	soln. E	—	100.0	—

† $cm^2\ volt^{-1}\ sec^{-1} \times (-1) \times 10^{-5}$

Table II

Electrophoretic Mobilities and Apparent Concentration Ratios of Component in Various Fractions of Cow Liver Catalase.

Component		1	2	3
Mobility† (mean value)		5.8	3.0	—
Apparent conc. ratio of comp. (%)	soln. B_1	18.5	81.5	(25.3)
〃	soln. D_1	16.7	83.3	(12.5)
〃	soln. F_1	—	100.0	—

† $cm^2\ volt^{-1}\ sec^{-1} \times (-1) \times 10^{-5}$

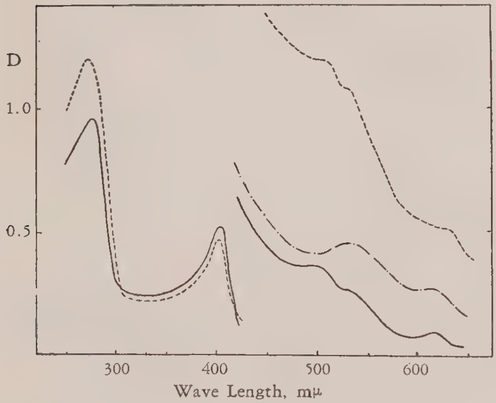

Fig. 7. Absorption Spectra of Various Fractions of Toad Liver Catalase in Phosphate Buffer (pH 7.0).

—·— Soln. B, ---- Soln. D, —— Soln. E:

cogen which has no electric charge at pH 7.0.

Absorption Spectra

The absorption spectra measured at each purification stage of toad liver catalase are shown in Fig. 7.

Solution B, the most impure solution in the tested samples, showed a large peak at 530 mμ in

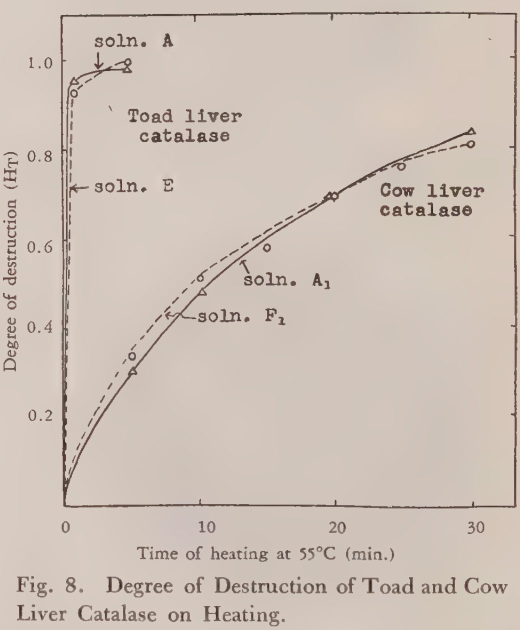

Fig. 8. Degree of Destruction of Toad and Cow Liver Catalase on Heating.

$$H_T = 1 - \frac{k'}{k}$$

H_T ---- Degree of destruction.
k ---- Velocity constant befor heating.
k′ ---- Velocity constant after heating.

Table III
Experimental Results of Catalase Purification from Toad Liver

Soln. Name	*Kat.-f.*	Electrophoretic Analysis.	Absorption Spectra.	Opt. Temp.
A	350	—	—	15°C
B	1100	3 components comp. 2, 53.2%	278, 405, 530, 620	15°C
D	8600	2 components comp. 2, 85.5%	278, 405, 510, 530, 630	15°C
E	21600	1 components comp. 2, 100%	278, 405, 510, 535, 615	15°C

Table IV
Experimental Results of Catalase Purification from Cow Liver.

Soln. Name	*Kat.-f.*	Electrophoretic Analysis.	Absorption Spectra.	Opt. Temp.
A_1	530	—	—	40°C
B_1	2300	2 components comp. 2, 81.5%	—	40°C
D_1		2 components comp. 2, 83.3%	—	40°C
F_1	39500	1 components comp. 2, 100%	276, 406, 502, 536, 627	40°C

the visible range. But when purification proceeded to the stage of solution D, namely, when component 3 was removed as shown in Fig. 5, absorption bands at 510 and 535 mμ, which were the characteristics of catalase and had been covered with large bands at 530 mμ in solution D, appeared.

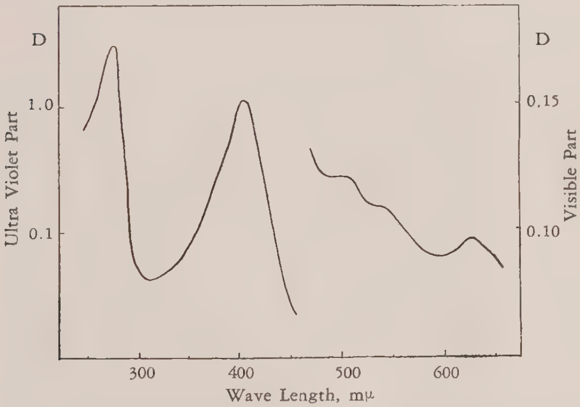

Fig. 9. Absorption Spectrum of Cow Liver Catalase.
Recrystallized crystal was disolved in distilled water containing a little amount of NaOH (pH 7.5).

The most purified catalase solution, then, shows five bands each centred at 278, 405, 510, 535, 615 mμ, which are closely resembled to the values of other catalases previously reported and also to the spectrum of our cow liver catalase as shown in Fig. 8.

Optimum Temperature

The optimum temperatures of catalase activities were measured on the same solution as what we used in the electrophoretic and spectrophotometric analysis. The results of these experiments are summarized in Tables III and IV.

As it is seen from Tables III and IV, catalase activity of toad liver (the cold blooded animal) has its optimum at 15°, but that of cow liver (the warm blooded animal) at 40°. These optimum temperatures correspond to those of their bodies.

Moreover, Tables III and IV show that these optimum temperatures of catalases are independent of enzyme purity. In other words, toad and cow liver catalases are different in their optimum temperature, and these differences are the same even if we measure such pure preparations as crystalline enzymes.

The difference of optimum temperatures between toad and cow liver catalases, may also suggest their difference in heat stability. The heat stabilities of those crystalline enzymes were measured, and the results are shown in Fig. 9. These results indicate that the difference of the optimum temperature is attributed to that of each enzyme protein itself.

In the following section we should like to discuss about this point in detail.

Discussion

From the absorption spectra of toad and cow liver catalases shown in Figs. 7 and 8, it is evident that the toad liver catalase like other animal catalases has the haemin as its prosthetic group.

On the other hand, it is doubtful whether the protein parts of cow liver and toad liver catalase are identical or not. We have often found such work dealing with the nature of enzyme proteins. In regard to catalases, some investigators indicated that the protein components of sheep, ox, horse and human catalase differ in their immunological nature[11, 12].

Morita and Kondo[13] once indicated the difference of electrophoretic mobilities in their studies on peroxidase from both Japanese radish and sweet potato, and discussed the nature of the enzyme proteins.

Judging from these reports, it is clear that the protein of the toad liver catalase is different from that of the cow liver catalase.

As it has been shown in Tables III and IV, the optimum temperatures measured with solutions of catalases extracted from cow liver and toad liver were different to each other, to such an extent that this difference, we thought, must have been due to the accompanied ions or the protective proteins extracted together with catalase. So, we measured the optimum temperatures of the pure, crystalline enzyme preparations which moved as a single boundary in electrophoretic analysis.

However, even in the case of such pure enzyme solutions, from which the accompanied ions and proteins other than catalase were excluded, the optimum temperatures of the solutions were found to be identical with those of the extracted solutions as shown in Tables III and IV; the activity of catalase from cow liver has its optimum at 40°, and that of the enzyme from toad liver at 15°.

From the experimental data obtained herein and discussion mentioned above, we must conclude that the optimum temperatures of the toad and the cow liver catalases are not identical, because of the dissimilarity of the enzyme proteins themselves.

In other words, the enzymes working in the circumstance of a higher temperature possess thermostable proteins, but on the contrary, the enzymes contained in lower temperature organisms are not so stable toward thermal destruction. These results are supported by Campbell[14] and Militzer[15] who report the same results as ours, dealing with several enzymes of thermophilic bacteria not in a crystalline state, but in a comparatively purified state.

To what parts of protein constitution cause this difference, is a problem to be investigated and should henceforth be solved in company with the progress of

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[11] E. Tria, *J. Biol. Chem.*, **129**, 377 (1939).

[12] D.H. Campbell and L. Fourt, *J. Biol. Chem.*, **129**, 385 (1939).

[13] Y. Morita and K. Kondo, *Bull. Res. Inst, Food. Science, Kyoto Univ.*, No. **13**, 60 (1954).

[14] L.L. Campbell, *Arch, Biochem. Biophys.* **54**, 154 (1955).

[15] W. Militzer and L. C. Tuttle, *Arch. Biochem. Biophys.* **39**, 379 (1952).

protein chemistry.

A careful study of the same kind of the enzyme isolated from several different sources may throw some considerable light on the nature of enzymic catalysis. Catalase, we think, must be a suitable enzyme for such investigations.

Summary

Catalase has been isolated from toad liver in a crystalline form. *Kat.-f.* of the crystal is 21600 and its crystalline form is a square thin plate.

A recrystallized crystal shows a single boundary in electrophoretic analysis, and its mobility is -2.8×10^{-5} cm^2/V sec. at pH 7.0.

A cow liver catalase isolated at the same time has a mobility of -3.1×10^{-5} cm^2/V sec. at pH 7.0.

The activity of crystalline toad liver catalase has its optimum at 15° and this temperature is in accordance with the optimum of the extracted solution, namely, the optimum temperature is constant even when the enzymes are purified to take crystalline forms.

The same experiments were made on the cow liver catalase and we have found that the optimum temperature of the cow liver catalase was 40° upon measuring both extracted solutions and crystalline enzymes.

These results show that the optimum temperatures of catalase are differ according to the sources from which they are extracted and that this difference is to be attributed to the protein parts of the enzyme molecules.

Acknowledgements

We wish to thank Mr. Morikazu Kajima, Atsushi Nakazawa and Miss Eiko Aburanokozi for their generous assistance.

Studies on Plant Catalase.

By Hisateru MITSUDA and Katsuharu YASUMATSU
Department of Agriculture, Kyoto University

Received August 11, 1955

Catalase activity is observed in almost all kind of plant material as far as it has been investigated.

The amount of catalase, however, is very low and the extracted enzyme solution very unstable, compared with that of animals.

From these reasons, the plant catalase has not yet been crystallized.

Now, we have succeeded in crystallizing the catalase extracted from both spinach and rice-plant leaves, by the application of the butanol-treatment to extract this from chloroplasts.

The crystal obtained has the same absorption spectrum as that of animal, and therefore it seems that plant catalase also has haemin as its prosthetic group.

Judging from their optimum temperatures, the protein part of both catalases seem to differ and each have a suitable optimum temperature in order to make its enzyme activity the highest in the circumstance where it grows.

The animal catalase was, as we have already shown in a previous paper, purified in a crystalline forms from several different sources.

But in sharp contrast with this, the enzymes of green leaves seemed to have been very difficult to crystallize and were thus left neglected by numerous investigators, because of their poorness in plants and the unstableness of extracted solutions.

For example, when the amount of plant catalase is compared with that of cow liver, the former is only about 1/25 as much as in the latter in spinach leaves, and 1/14 in rice-plant leaves when calculated as: $\frac{\text{total catalase activity}}{\text{g. of material used}}$.

In other words, if we want to obtain the same amount of plant catalase as animal catalase, for its purification, we must use a quantity 25 times as much as animal sources for spinach leaves and 14 times for rice-plant leaves to attain this purpose. As the results of the poorness of plant catalase shown above and the lack of stability of the extracted catalase solution, our knowledge on the nature of these enzymes has been very little.

In 1942, one of us[1)] succeeded in crystallizing the enzyme from rice-plant leaves, using the dioxane method with which Sumner and Dounce[2)] crystallized animal catalase for the first time. But as dioxane is too dear a solvent to be used for enzyme purification in Japan, we have consequently been trying to crystallize the plant catalase without the use of dioxane.

In other papers[3, 6)], we reported that

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1) H. Mitsuda: Published in the J. of Agricultural Chemical Society of Japan (May, 1942), and in the lecture at the Institute for Chemical Research, Kyoto Univ. (Nov. 1943), H. Mitsuda: *Bull. Agr. Chem. Soc. Japan* **18** B, 35 (1942) H. Mitsuda: Studies on Vitamin C and Phytocatalase (1947) H. Mitsuda: *J. Japanese Soc. Food and Nutrition*, **3**, 7 (1950).

2) J.B. Sumner and A. L. Dounce, *J. Biol. Chem.*, **121**, 417 (1937).

3) H. Mitsuda and A. Nakazawa, *Bull. Inst. Chem. Res., Kyoto Univ.*, **32**, 19 (1954)

the phosphate buffer was the best stabilizer of catalase and the butanol treatment, adopted by Morton[4] to separate enzymes associated with insoluble particles, was very suitable to extract catalase from chloroplasts. Then, we have succeeded again in crystallizing plant catalase from both spinach and rice-plant leaves, by using this improved method.

We now report, in this paper, the method of purification and also on some enzyme properties, hoping to receive comments and criticism from the readers.

Method

The activity of catalase preparations was measured according to Euler and Josephson's method[5].

The purity of an enzyme preparation is expressed as:

$$\textit{Kat.-f.} = \frac{\text{velocity constant}}{\text{g. enzyme used in the test}} .$$

Optimum temperatures were measured according to the method mentioned in the previous paper.

Absorption spectra was observed with a Beckmann Model DU spectrophotometer.

Experimental Results

Isolation of Crystalline Enzyme

In Fig. 1, the method used for the purification of spinach leaf catalase is shown.

The total volume of the extracted solution (solution A_2) was approximately 15.5 l and had a *Kat.-f.* of about 14. This *Kat.-f.* is very small compared with that of animal catalase as shown in the last paper, i.e., the *Kat.-f.* of toad liver is 350 and that of cow liver 530.

N-Butanol was added to this extracted solution, to extract catalase from chloroplasts and to make atrue solution. As will be seen from Table I, the yield of this treatment was 78%.

It is clear that the association of an enzyme with a certain particle is an unmitigated nuisance

Solution Name — **Leaf-blade of Spinach** (13 kg)

wash, cut
homogenize with 10 l phosphate buffer (1/60 M. pH 7.0)
press with cotton clothes

A_2 Green Suspension 15.5 l

cool to 4°
6.2 l cold *n*-butanol (0.4 volume)
stir. 1 hour at 4°
stand in an ice-box for 1 night
separate clear aqueous layer by siphon

B_2 Yellowish Clear Solution 13.5 l

cool to 3°
10.8 l cold acetone (0.8 volume)
stir. 20 min. stand in an ice-box for 1.5 hr.
centrifuge 3000 r. p. m. 5 min.

Precipitate

phospate buffer (1/100 M, pH 7.0) 1 l
stir, 30 min.
stand in an ice-box for 1 night
centrifuge 3000 r. p. m. 1 hr.

C_2 **Supernatant 1000 ml.**

cool to 4°
cryst. $(NH_4)_2SO_4$ 352 g (to 50% sat).
centrifuge 3000 r. p. m. 30 min.

Precipitate

260 ml. distd. water
centrifuge 3500 r. p. m. 1 hr.

D_2 **Supernatant 300 ml.**

sat. $(NH_4)_2SO_4$ soln. 40 ml. (to 11.8% sat.)
centrifuge

Supernatant

sat. $(NH_4)_2SO_4$ soln. 160 ml. (to 40% sat.)
centrifuge 4000 r. p. m. 20 min.

Precipitate

50 ml. distd. water
centrifuge 3500 r. p. m. 30 min.

E_2 Supernatant 50 ml.

sat. $(NH_4)_2SO_4$ soln. 10 ml. (to 16.7% sat.)
centrifuge 3500 r. p. m. 1 hr.

Supernatant

sat. $(NH_4)_2SO_4$ soln. 8 ml. (to 27% sat.)
centrifuge 3500 r. p. m. 30 min.

Precipitate

6 ml. distd. water
centrifuge 3500 r. p. m. 30 min.

F_2 Supernatant

stand in an ice-box for 2 days

Silkiness with stirring

Needle Crystals

Fig. 1. The Method to Prepare the Crystalline Spinach Leaf Catalase.

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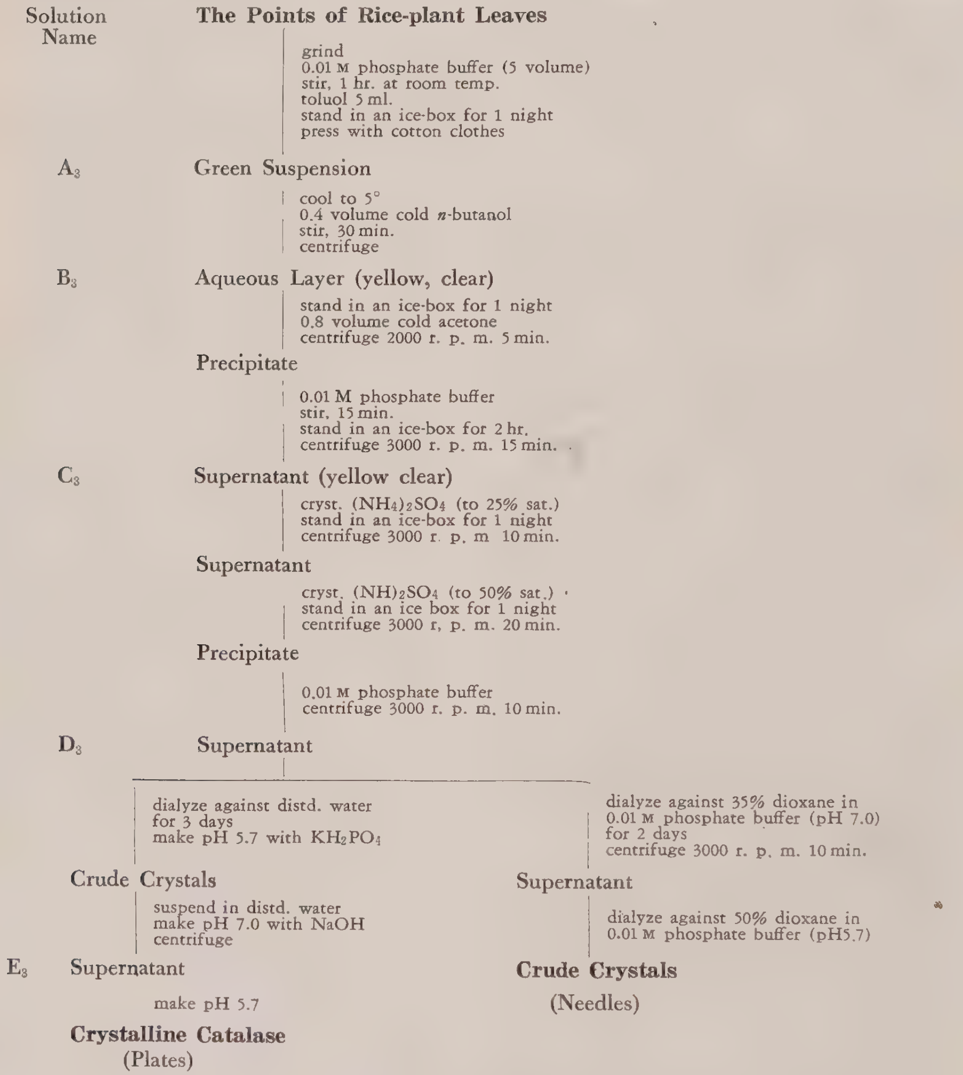
[4] R.K Morton, *Nature*, **166**, 1092 (1950).

[5] H.v. Euler, and K. Josephson, *Ann.*, **452**, 158 (1927).

Table I Purification of Catalase from Spinach Leaves.

Soln. Name	Volume (ml.)	Total Activity	*Kat.-f.*	Yield (%)	Purification	Opt. Temp.
A_2	15500	34000	14	100	1	15°C
B_2	13500	26500	70	78	5	15°C
C_2	1000	24100	180	71	13	15°C
D_2	300	20400	2200	60	153	15°C
E_2	50	14700	3300	43	230	15°C
F_2	6	—	15300	—	1065	15°C



Solution Name

The Points of Rice-plant Leaves

grind
0.01 M phosphate buffer (5 volume)
stir, 1 hr. at room temp.
toluol 5 ml.
stand in an ice-box for 1 night
press with cotton clothes

A_3 Green Suspension

cool to 5°
0.4 volume cold *n*-butanol
stir, 30 min.
centrifuge

B_3 Aqueous Layer (yellow, clear)

stand in an ice-box for 1 night
0.8 volume cold acetone
centrifuge 2000 r. p. m. 5 min.

Precipitate

0.01 M phosphate buffer
stir, 15 min.
stand in an ice-box for 2 hr.
centrifuge 3000 r. p. m. 15 min.

C_3 Supernatant (yellow clear)

cryst. $(NH_4)_2SO_4$ (to 25% sat.)
stand in an ice-box for 1 night
centrifuge 3000 r. p. m 10 min.

Supernatant

cryst. $(NH)_2SO_4$ (to 50% sat.)
stand in an ice box for 1 night
centrifuge 3000 r. p. m. 20 min.

Precipitate

0.01 M phosphate buffer
centrifuge 3000 r. p. m. 10 min.

D_3 Supernatant

dialyze against distd. water
for 3 days
make pH 5.7 with KH_2PO_4

Crude Crystals

suspend in distd. water
make pH 7.0 with NaOH
centrifuge

E_3 Supernatant

make pH 5.7

Crystalline Catalase
(Plates)

dialyze against 35% dioxane in
0.01 M phosphate buffer (pH 7.0)
for 2 days
centrifuge 3000 r. p. m. 10 min.

Supernatant

dialyze against 50% dioxane in
0.01 M phosphate buffer (pH5.7)

Crude Crystals
(Needles)

Fig. 2. The Method to Prepare the Crystalline Rice-plant Leaf Catalase.

Table II
Purification of Catalase from Rice-plant Leaves.

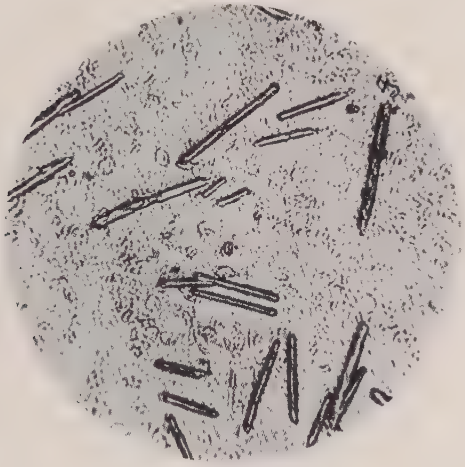
Soln. Name	Volume (ml.)	Total Activity	*Kat.-f.*	Yield (%)	Purification	Opt. Temp.
A_3	4400	6900	28	100	1	35°C
B_3	4100	4670	40	68	1.5	35°C
C_3	222	2600	430	37	16	35°C
D_3	10	1200	2260	18	81	35°C
E_3	2	—	7120	—	250	35°C

when isolating it in a highly purified stage. To readily obtain the catalase solution from chloroplasts with a high yield is, then, a very important but difficult object. From this view-point, we consider the butanol treatment to be a very suitable method for this purpose. Following the above treatment the method corresponds to that of animal catalase.

The method applied for purification of rice-plant leaf catalase is shown in Fig. 2 and its results are given in Table II.

Only points of rice-plant leaves at the flowering period, were used for this purpose, as catalase activity was found to be highest in this part and period.

The amount of the rice-plant leaf catalase is larger than that of spinach catalase, therefore the *Kat.-f.* of the rice-plant extracted solution is about two times as large as that of spinach. The *Kat.-f.* 28 is, however, smaller than that of animals.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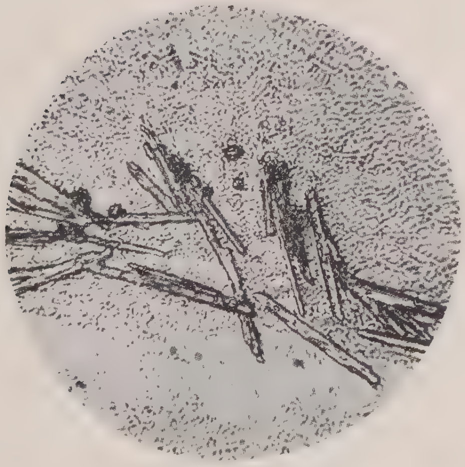
b

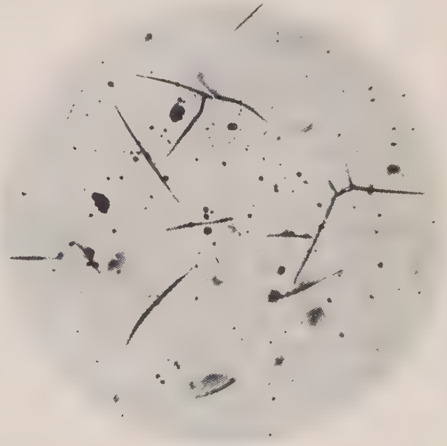
c

Fig. 3. Crystalline Catalase from Rice Plant Leaves.

a, b ---- Crystallized from Soln. E_3 (×1000)
c ---- Crystallized by Dialysis (×200)

For purification the same method as spinach was used.

Fig. 3 shows a crystalline catalase extracted from green leaves of rice-plant.

Absorption Spectrum

In Fig. 4, the absorption spectrum of spinach leaf catalase (*Kat.-f.* 15300) is illustrated, it shows peaks at 278 and 405 mμ and shoulderes at 505, 540 and 620 mμ, resembling that of animal catalases.

Optimum Temperature

We measured the optimum temperature of crystalline catalase from the view-point reported in the previous paper.

Namely, from our measurements of the extracted solutions, the optimum temperature of the rice-plant leaf catalase (this plant grows in summer) is 35°, but that of the spinach leaf catalase (this vegetable grows in winter) is 15°. These optimum temperatures closely resemble the temperature in which they grow. Now, as we prepared the crystalline catalases from both spinach and rice-plant, we proceeded to measure their optimum temperatures.

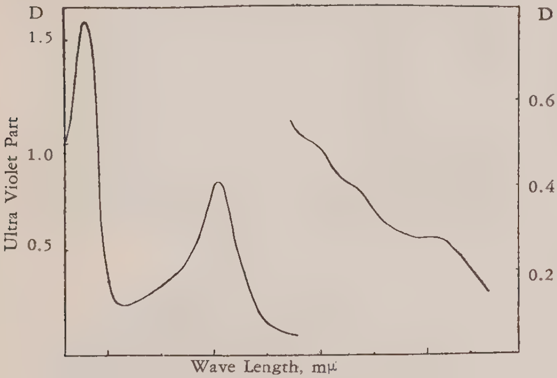

Fig. 4. Absorption Spectrum of Spinach Leaf Catalase. (*Kat.-f.* 15300)

As it is shown in Tables 1 and 2, the optimum temperature is independent of enzyme purity.

Discussion

In order to separate the plant catalase associated with chloroplasts, the *n*-butanol treatment which was originally adopted for purification of the alkaline phosphatase[4], was adopted here. This treatment is specially suitable for dissociating either lipid-protein complexes or protein-protein complexes.

Among the several different methods formerly investigated[6], this butanol treatment is considered to be the best for catalase extraction. We think this treatment is to be most suitable for the purification of plant catalase when a large amount of the sample is used, because when the catalase is mixed with the butanol and kept at 0°, the catalase activity did not decrease at all for a whole week.

Kondo, Chiba and Kawai[7] once reported that when they had isolated plant carbonic anhydrase from the spinach leaves, they found no trace of zinc in the purified plant enzyme, though zinc had always been found in the animal enzyme. Namely, it is conceivable that, even among the purified enzymes which catalyze the same reaction, some differences exist according to their sources.

It is needless to say that the animal catalase has haemin as its prosthetic group and catalase is a sort of haemeprotein such as the haemoglobin in the blood of the animal. The sole difference of the protein part makes both the catalase and the haemoglobin specific in their physiological actions, i.e., the catalase catalyzes the decomposition of peroxide and the haemoglobin carries the molecullar oxygen.

But in green leaves, the chlorophyll which has a porphyrin ring resembling haemoglobin is found in the chloroplasts, and it plays a physiologically important part. Moreover, the catalase is also found in the chloroplasts.

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[6] H. Mitsuda and K. Yasumatsu, *Bull. Inst. Chem. Res. Kyoto Univ.*, **33**, 136 (1955).

[7] K. Kondo, H. Chiba and F. Kawai, *Bull. Res. Inst Food Science, Kyoto Univ.*, No. **8**, 17 (1952).

Judging from the fact that the plant catalase shows the same absorption spectrum as the animal one, we consider the plant catalase to have haemin as its prosthetic group. Herbert and Pinsent[8] report that the crystalline bacterial catalase also has haemin as its prosthetic group. Accordingly, we think it would be clear that the prosthetic group of plant catalase is the haemin.

In contrast to the identity of the prosthetic group of the animal catalase and the plant catalase, the protein parts seem to be reciprocally different. In the previous paper we have reported the difference of the optimum temperature of animal catalases and have discussed the nature of protein parts of the enzyme.

We can discuss the plant catalase in the same manner as the animal catalase. The optimum temperature of the plant catalase resembles to the temperature of the circumstances where the plant grows, and is independent of enzyme purity as we have shown in Tables I and II.

From the results obtained from the measurements of the optimum temperature, it may be suggested as follows: (1) the plant adapts itself to the circumstances where it grows, by making most active enzymes suitable to their own temperature, i.e., by changing the optimum temperature of their enzymes. (2) these differences are not due to accompanied ions or protective proteins but to the enzyme protein itself.

We are now also studying the physical and plant physiological properties of the catalase, too.

Summary

We purified the catalases of both the spinach leaves and rice-plant leaves by the *n*-butanol treatment and isolated them into crystalline forms.

The absorption spectrum of the spinach leaf catalase is identical with that of the animal catalase.

The activity of the spinach leaf catalase has its optimum at 15°, but that of the rice-plant leaf catalase at 35°.

These optimum temperatures have resemblance to the circumstances where they grow and are independent of enzyme purity.

Acknowledgements

We wish to thank Mr. Morikazu Kajima, Atsushi Nakazawa and Miss Eiko Aburanokozi for their generous assistance.

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Separation and Identification of Fatty Acids.

Part XVII. Paper Chromatography of Saturated Fatty Acids as Their 2,4-Dinitrophenylhydrazides[1]

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Received August 15, 1955

An improved method is described for separating saturated fatty acids by reversed-phase paper chromatography. The 2,4-dinitrophenylhydrazides prepared from saturated fatty acids from C_2 to C_{22} are run upward on paper impregnated with tetralin, using 90% methanol—acetic acid—tetralin, 80% ethanol—acetic acid—tetralin, or 90% methanol—tetralin as the moving solvent. The simultaneous separations of all even-carbon acids from C_6 to C_{22}, all odd-carbon acids from C_7 to C_{19}, and also all odd- and even-carbon acids from C_7 to C_{19} can be successfully performed by means of this paper chromatography. The method is useful for the detection of component saturated fatty acids in natural fats.

Although the reversed-phase paper chromatography has been successfully employed for the separation of longer-chain fatty acids and their derivatives by several workers[2-9], the technique has not been quite sufficient for achieving clean separation of the component fatty acids in natural fats with complex composition, owing to its relatively lower efficiency. In order to improve the separability and sensitivity by this reversed-phase method, a convenient method was proposed in a previous paper[10], by which unsaturated acid esters could be selectively detected as their mercuric acetate addition compounds. The present investigation has been undertaken with the intention of finding a satisfactory method for paper-chromatographic separation of saturated fatty acids, capable of combining with paper chromatography of unsaturated acid esters by the mercuration method. For this purpose, 2,4-dinitrophenylhydrazides of saturated fatty acids, of which only a few have hitherto been reported in the literatures[11, 12, 13], have been used as the derivatives for paper chromatography, and they have displayed excellent separability on the chromatograms. The preparation, properties, and paper-chromatographic separation of 2,4-dinitrophenylhydrazides of saturated fatty acids and the application of this chromatographic technique to the analysis of the component saturated acids of natural fats are reported in this paper.

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Experimental

Fatty Acids.

The even-numbered acids from acetic to stearic were all commercial preparations, which were completely purified by redistillation, recrystallization, or chromatography. Arachidic acid was prepared from stearyl alcohol by malonic ester synthesis. Behenic acid was derived from erucic acid by hydrogenation. The odd-numbered acids from heptanoic to heptadecanoic were prepared by hydrolysis of the corresponding nitriles obtained from the amides of the next higher fatty acids by Hofmann degradation, respectively. Nonadecanoic acid was prepared from stearyl alcohol by nitrile synthesis. These fatty acids were found of high purity by the determination of their physical and chemical constants and also by single chromatographic experiments.

Preparation of 2,4-Dinitrophenylhydrazides.

Fatty acid was converted into its acid chloride by the action of thionyl chloride. From 10 to 20% excess of the acid chloride was added dropwise to a 10% solution of 2,4-dinitrophenylhydrazine in dry pyridine under cooling and shaking. If the reaction mixture was not homogeneous, it was slightly warmed for a moment until any precipitate disappeared, and then allowed to cool. After twenty minutes, a large quantity of water was poured into the mixture, and the crude 2,4-dinitrophenylhydrazide was collected by filtration followed by washing with water and then with petroleum benzine. The yield was 85–94%. Recrystallization from alcohol and benzene and subsequent washing with petroleum benzine gave the pure compound as yellow crystals. The derivatives of arachidic and behenic acids were purified further by the use of paper chromatopacks, a methanol—acetic acid—tetralin system being employed.

In the case of the caproic or caprylic acid derivative, benzene was added to the reaction mixture, and the benzene extract thoroughly washed free from pyridine with hot water. The crude product which crystallized from the benzene layer on cooling, was collected, washed with petroleum benzine, and recrystallized.

The 2,4-dinitrophenylhydrazides of acetic and butyric acids were also prepared by heating the corresponding fatty acid anhydrides with 2,4-dinitrophenylhydrazine and recrystallizing the consequent products from alcohol.

The melting points, solubilities in various solvents, and analytical data of these derivatives are listed in Table I.

The crystals of 2,4-dinitrophenylhydrazides were dissolved in benzene or alcohol, each containing a small amount of pyridine, and subjected to paper-chromatographic analysis to determine their R_F values.

Simplified Method of Preparation for Paper Chromatography.

A mixture of 10 mg. of fatty acids and 0.3 ml. of thionyl chloride was refluxed for thirty minutes, and the excess of thionyl chloride was then removed by distillation in vacuo. To the reaction product, 0.1 ml. of a 5% solution of 2,4-dinitrophenylhydrazine in dry pyridine was added with cooling. After being briefly warmed to become homogeneous, the mixture was allowed to cool to room temperature for twenty minutes. This solution could be directly used as the sample solution for paper chromatography, but in the case of higher fatty acids, it was preferable to use the upper benzene layer for paper-chromatographic analysis, after diluting the solution with 2 ml. of benzene and adding 10–20 ml. of warm water with shaking.

The procedure was satisfactorily applicable to more than 2 mg. of the original fatty acids, the amounts of reagents being varied in proportion to those of fatty acids.

Paper Chromatography.

From 0.5 to 3 μl. of the sample solutions, containing 2–100 μg. of each 2,4-dinitrophenylhydrazide, were placed at individual points of origin along a horizontal line ruled 5 cm. from

Table I

Physical Properties and Analyses of 2,4-Dinitrophenylhydrazides of Fatty Acids.

Acid	M.p., °C.	Solubility[a] A	Ch	P	Ea	B	E	M	Et	Ct	Pe	W	N, % Calcd.	Found
Acetic[b]	199.5	++++	±	++++	++	−	++	++	+	−	−	+	23.33	23.17
Butyric	168	++++	++++	++++	++++	+	+++	++	+	±	−	+	20.89	20.81
Caproic	112	++++	++++	++++	++++	+	+++	++	++	±	−	±	18.91	18.82
Caprylic	102	++++	++++	++++	++++	++	++	++	+++	±	−	±	17.28	17.46
Capric	113.5	++++	++++	++++	+++	++	++	++	++	±	−	−	15.90	15.78
Lauric[b]	116[c]	+++	+++	+++	+++	++	++	+	++	±	−	−	14.73	14.89
Myristic[b]	118	+++	+++	+++	+++	++	+	+	++	±	±	−	13.72	13.78
Palmitic[b]	120	++	++	+++	++	++	+	+	+	+	±	−	12.83	12.95
Stearic[b]	122	++	++	++	++	++	+	+	+	+	±	−	12.06	12.13
Arachidic	123.5	++	+	++	++	++	+	+	+	+	±	−	11.37	11.14
Behenic	124	++	+	+	++	++	+	+	+	+	±	−	10.76	10.60

[a] At root temperature. Solvents: A, acetone; Ch, chloroform; P, pyridine; Ea, ethyl acetate; B, benzene; E, ethanol; M, methanol; Et, ether; Ct, carbon tetrachloride; Pe, petroleum ether; W, water. Solubilities are indicated by the scale; ++++ very soluble to − insoluble.

[b] Previously prepared.[11,12,13)]

[c] Reported m.p. 110-111 °C.[12,13)]

Table II

R_F Values of 2,4-Dinitrophenylhydrazides of Fatty Acids[a].

Acid	No. of C atoms in acid	Solvent system[b] MAT	EAT	MT
Acetic	2	0.85	0.93	0.77
Butyric	4	0.88	0.97	0.82
Caproic	6	0.83	0.93	0.81
Heptanoic[c]	7	0.78	0.90	0.78
Caprylic	8	0.74	0.87	0.74
Nonanoic[c]	9	0.69	0.81	0.68
Capric	10	0.64	0.78	0.64
Undecanoic[c]	11	0.57	0.72	0.56
Lauric	12	0.54	0.70	0.51
Tridecanoic[c]	13	0.45	0.62	0.43
Myristtic	14	0.42	0.60	0.38
Pentadecanoic[c]	15	0.34	0.52	0.30
Palmitic	16	0.30	0.49	0.25
Heptadecanoic[c]	17	0.25	0.41	0.19
Stearic	18	0.21	0.38	0.15
Nonadecanoic[c]	19	0.16	0.30	0.12
Arachidic	20	0.13	0.27	0.09
Behenic	22	0.08	0.18	0.05

[a] Ascending chromatography at 30°C.

[b] Solvent systems: MAT, methanol—acetic acid—tetralin; EAT, ethanol—acetic acid—tetralin; MT, methanol—tetralin.

[c] 2,4-Dinitrophenylhydrazides of odd-numbered acids were obtained as crystals individually and used for paper chromatography without recrystallization.

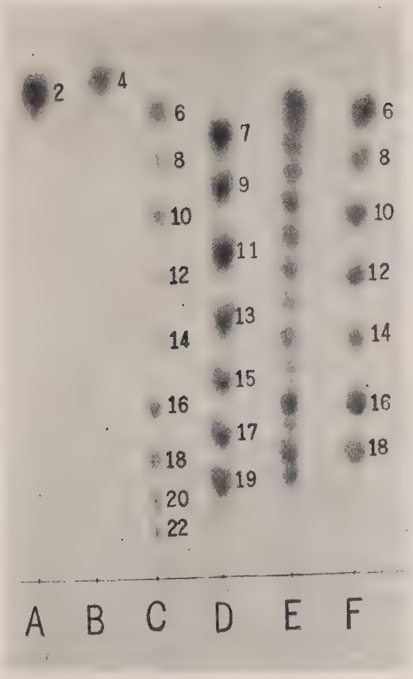

Fig. 1. Paper chromatogram of the 2,4-dinitrophenylhydrazides of saturated fatty acids.
Solvent system: MAT. The number beside a spot represents the number of carbon atoms in the acids. E and F were prepared by the simplified method from the mixed fatty acids: E, all odd- and even-numbered acids from C_6 to C_{19}; F, all even-numbered acids from C_6 to C_{18}.

the lower edge of a large sheet of Toyo No. 2 filter paper. The paper was uniformly sprayed four times repeatedly with tetralin, the stationary solvent; this required approximately 0.45 ml. of tetralin per 100 cm². of the paper. The impregnated paper was immediately wound round the glass coils of a paper holder, and it was then placed in a glass cylinder maintained at 30°C. in a canstant-temperature room and developed by the ascending technique. The developing solvents used were 90% ($^v/_v$) methanol—acetic acid—tetralin (10 : 2 : 1 by volume) (MAT), 80% ethanol—acetic acid—tetralin (10 : 2 : 1) (EAT), and 90% methanol—tetralin (10 : 1) (MT). A development time from five to six hours was suitable for complete separation of the compounds, the distance of development from the starting line reaching 18–26 cm., depending upon the solvent used. The paper was then removed from the cylinder, dried overnight at room temperature, and sprayed with a 0.5 N solution of potassium hydroxide in alcohol. The 2,4-dinitrophenylhydrazides were detected by spraying as brown spots with high sensitivity, although they were visible as yellow spots even before spraying.

The R_F values of the 2,4-dinitrophenylhydrazides of both even- and odd-numbered fatty acids, from acetic to behenic, are given in Table II. Figures 1 and 2 show two paper chromatograms illustrating the separability of the compounds.

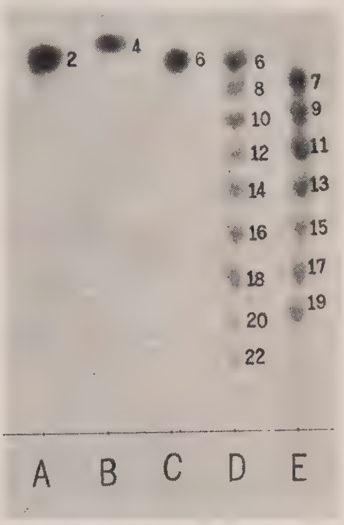

Fig. 2. Paper chromatogram of the 2,4-dinitrophenylhydrazides of saturated fatty acids.
Solvent system: EAT. The number beside a spot represents the number of carbon atoms in the acid.

Analysis of the Component Saturated Acids of Natural Fats.

The application of this paper-chromatographic technique to the detection of the component acids of natural fats was attempted. The mixed fatty acids obtained from coconut oil, Japan wax, beef tallow, and peanut oil were used as the samples for these experiments. A sample of 10 mg. of the mixed fatty acids was converted into a mixture of their 2,4-dinitrophenylhydrazides by the above simplified method of preparation and chromatographed on paper with the solvent systems MAT and EAT. The results of the

Table III

Separation and Identification of the Component Saturated Acids of Natural Fats by Paper Chromatography.

Fat	Coconut oil	Japan wax	Beef tallow	Peanut oil
A. V.	4.1	9.2	2.8	0.6
Sap. V.	258.5	208.7	197.7	190.3
I. V.	8.3	13.9	45.8	98.4
Acid	R_F values of spots detected[a]			
Caproic	0.82 vw	—	—	—
Caprylic	0.74	—	—	—
Capric	0.63	—	—	—
Lauric	0.53 vs	—	—	—
Myristic	0.41 s	0.42 vw	0.42	0.41 vw
Palmitic	0.30	0.31 vs	0.30 vs	0.30 vs
Stearic	0.21	0.22 vs	0.21 vs	0.21 vs
Arachidic	0.14	0.13	0.14 vw	0.13
Behenic	0.09 vw	—	—	0.08
Lignoceric	—	—	—	0.04[b]vw

[a] Ascending chromatography at 30°C. Solvent system: MAT. Color intensity of spots: vs, very strong; s, strong; vw, very weak.

[b] Lignoceric acid could be identified more distinctly with the system EAT.

separation on the chromatograms are summarized in Table III.

Results and Discussion

It was found that the 2,4-dinitrophenylhydrazides of saturated acids possess sharp melting points and a sufficient temperature increment between adjacent members to render them also valuable for purposes of characterizing fatty acids as their crystalline derivatives, although it was somewhat difficult in the cases of the fatty acid derivatives higher than stearic to purify the derivatives completely by recrystallization alone.

Solubility data of the derivatives roughly estimated (Table 1) were useful in selecting the solvents for recrystallization and chromatography. It was also found that the solubility of the derivatives in each solvent varied with the chain length of fatty acid, the variation being very characteristic of the solvent.

The paper-chromatographic separation of these compounds from each other is perfect, because they slightly diffuse on paper during development and therefore give small distinct spots. It seems that tetralin, as the stationary solvent, may be suitable for the separation of these aromatic derivatives of fatty acids. The best separation is attained with the solvent system MAT, but the system EAT is also effective especially for the separation of the fatty acids higher than arachidic acid. By using the system MAT, a mixture of 2,4-dinitrophenylhydrazides of all odd- and even-numbered acids from heptanoic to nonadecanoic could be separated from each other on a chromatogram (Fig. 1).

The R_F values are affected by various conditions, e.g., temperature, amount of the sample, volume of the stationary solvent, etc. Although a constant temperature is maintained during development, the R_F values have a tendency to vary to some extent with atmospheric temperature; the higher the atmospheric temperature, the lower are the R_F values. The R_F values given in Tables II and III are measured at a developing temperature of 30°C. and an atmospheric temperature of 10–15°C. The optimum amount of the sample to be applied ranges from 2 to 100 μg. of each 2,4-dinitrophenylhydrazide which retains a constant R_F value, as far as it is applied within this range.

The derivatives of unsaturated acids give intense spots near the original points when they are prepared by the above simplified method. This may probably be due to the decomposition and polymerization of unsaturated acids and their derivatives during chlorination and subsequent condensation. Only when the chlorination was carried out under mild conditions, such as at room temperature, oleic acid yielded together with other byproducts its own 2,4-dinitrophenylhydrazides, the R_F values of which were 0.24 with MAT and 0.42 with EAT. Therefore, in the analysis of the component acids of natural fats described above, no distinct spot resulting from the unsaturated acids is to be observed except in the vicinity of the original points, which does not practically interfere with detecting the spots of common saturated acids. The preliminary removal of unsaturated acids by the lead salt—alcohol method is effective for the identification of some saturated higher acids, such as lignoceric acid in peanut oil, which, otherwise, are liable to be missed among the intense spots resulting from unsaturated acids near the original point. The effect of excess 2,4-dinitrophenylhydrazine on the chromatogram is also negligible, because it gives a very diffuse and much less sensitive spot, on spraying with potassium hydroxide solution.

From the results of paper-chromatographic analysis of natural mixed acids, it was observed that intensities and areas of the spots on the chromatogram were roughly proportional to the amounts of the corresponding component saturated acids in natural fat, respectively though their spots showed a somewhat increasing intensity along with the increase in the number of carbon atoms in the acids, i.e., a relatively intense coloration of higher fatty acids in comparison with their actual amounts. The method is particularly excellent in detecting the minor component acids. In these experiments, behenic acid in coconut oil, formerly unidentified, has been detected as the minor component acid (Table III).

It appears that this method provides an effective means of separation and identification for saturated higher fatty acids and should be applicable to the simple microanalysis of natural mixed fatty acids.

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